METABOLIC METHODS

Clinical Procedures in the Study of Metabolic Functions

By

C FRANK CONSOLAZIO

Chief of Biochemistry United States Army
Medical Nutrition Laboratory
Chicago Illinois

ROBERT E JOHNSON, M D, D Phil (Oxford)

Professor and Head of the Department of Physiology
University of Illinois Urbana Illinois

and

EVELYN MAREK, M A

Biochemist, United States Army
Medical Nutrition Laboratory
Chicago Illinois

ILLUSTRATED

ST LOUIS
THE C V MOSBY COMPANY
1951

Press of
The C V Mosby Company
St Louis

THIS BOOK IS DEDICATED
TO OUR COLLEAGUES PAST AND
PRESENT AT THE HARVARD FATIGUE
LABORATORY AND THE MEDICAL
NUTRITION LABORATORY

PREFACE

This book presents in detail methods which have proved to be useful in our experience during fifteen years of research on various aspects of mammalian metabolism particularly that of human beings in health and in disease Three somewhat different types of work have posed methodological problems first metabolic balance studies second routine laboratory analysis of tissues food and body fluids and third studies out in the field A common characteristic of all three is the accumulation of large numbers of specimens on which a wide variety of analyses must be performed The most satisfactory methods in all three cases are those which use the least amount of samples and which can be run routinely in large numbers Sometimes it is desirable to sacrifice some specificity in favor of simplicity, and sometimes it is necessary to sacrifice some accuracy for the sake of speed and reproducibility We have described in detail only those methods which have been used routinely by us long enough to clarify the precautions essential for success

We have omitted intentionally any description of isotopic ultracentrifuge and electrophoretic techniques for the reason that as yet they are not widely used routinely in metabolic balance studies This omission will doubtless have to be rectified in future editions

The manner of presentation adopted in this book has evolved over many years of training technicians and graduate students We have found that a uniform detailed presentation following a single basic outline is a great saving of time for the instructor especially when examples of calculations and a listing of important precautions are also included

Some of the material in this book was taken from two previous compilations

Syllabus of Laboratory Methods from the Harvard Fatigue Laboratory " by W V Consolazio S M Horvath and D B Dill (1941) and

Laboratory Manual of Field Methods for Biochemical Assessment of Metabolic and Nutritional Condition" by R E Johnson F Sargent C F Consolazio and P F Robinson (1945)

We are of course indebted to many people for help and advice during the preparation of this book Our former colleagues D B Dill H S Belding R C Darling and F Sargent have given valuable assistance We are particularly indebted to Mr Carl Gordon for producing or redrawing all of our charts Almost all members of the Medical Nutrition Laboratory helped at one time or another especially the whole secretarial staff In particular Mrs I DeCordial worked hard in preparing many drafts and Mrs L Vrhel

Mrs I DeCordial and Miss E Bielat were solely responsible for the final typography of the book Members of the Statistical Branch assisted in many ways and Captain J Rielly MSC rendered great service in the assembling of the manual The members of the Biochemistry Branch (Dr G Weigend Mr J Bell Corporal T Hutton Corporal R Powell and Private R Golden) helped out on many occasions and Private H Rosen drafted the sections on the Beckman spectrophotometer and flame photometer The section on micro biological procedures was prepared by Captain L Crowley MSC Mrs B Enwall and Miss L Blanksma Miss M Maloney assisted in the preparation of the sections on clinical laboratory procedures

In a book such as this most of the work is not original Permission has been obtained from authors and publishers for the reprinting of certain charts and graphs For the use of illustrations of apparatus we wish to thank Dr Peter Scholander Swarthmore University Pennsylvania Dr Ancel Keys Laboratory of Physiological Hygiene University of Minnesota Mr W V Consolazio Office of Naval Research Washington D C Dr D B Dill Medical Division Edgewood Arsenal Maryland and Dr H S Belding of the Climatic Research Laboratory Lawrence Mass

We have received willing permission from numerous manufacturers to describe their products and have been supplied by them with photographs of their instruments and with circuit diagrams In the appropriate places in the text their contributions are acknowledged

C F Consolazio
R E Johnson
Evelyn Marek

Urbana Illinois and
Chicago Illinois

CONTENTS

METABOLIC METHODS

Clinical Procedures in the Study of Metabolic Functions

SECTION 1

INTRODUCTION

The purpose of this manual is to assist in the carrying out of metabolic studies whether in the laboratory or in the field Perhaps a brief description of how the book is organized will facilitate its use

A standard form of presentation has been adopted for all detailed descriptions of methods The major headings presented in each case are (a) title of method (b) a key reference (c) a brief outline of the principle of the method (d) a detailed list of apparatus that will be required (e) a detailed list of reagents and how to prepare them (f) a step by step description of the method (g) the general equation for calculating the results (h) an example of a typical set of results calculated according to the general equation and (i) precautions which we have found to be important in using the method Those sections that do not deal with specific methods have no fixed pattern the presentation being appropriate to the subject matter

The first major section of the book deals with the collection and storage of specimens this being a primary concern in all metabolic studies The second major section deals with instrumentation and includes detailed descriptions of those instruments which will be used in the various procedures presented later in the book The third main section presents biochemical procedures covering the categories of minerals protein and nitrogen compounds carbohydrates and derivatives fats and derivatives vitamins hormones enzymes pigments and hydrogen ion concentration The fourth section deals with microbiological procedures which are applicable to routine analysis of vitamins and amino acids The fifth section is concerned with physiological measurements blood gases physical chemistry of blood gases estimation of respiratory gases measurement of respiratory metabolism pulmonary measurements and the estimation of plasma volume blood volume and extracellular fluid

Starting with the sixth major section the presentation changes somewhat The sixth section itself deals with studies in the field in which the authors have had a wide and varied experience (Field physiology poses many interesting methodological problems which have to be met in a manner not always as rigorous as is customary in the laboratory) The seventh section discusses briefly some of the techniques which we have found useful in metabolic wards The last major section deals with clinical laboratory procedures emphasis being placed on functional tests This section does not attempt to be comprehensive in fields of clinical pathology that have been covered well in numerous other textbooks

Two minor sections finish the text of this manual A list of references is given to standard statistical treatises Finally a series of miscellaneous tables of useful data is appended

Throughout the book extensive use is made of D Ocagne alignment charts We have found that these charts have two great virtues in metabolic studies first they speed up the computation of the voluminous data which are always collected, second they minimize errors in computation. All of the alignment charts presented herein are used in the same way the final answer is read from the middle line when a string or ruler is stretched straight be tween the values listed on the right and left hand lines

Since no two workers will always agree on the merits of individual methods we have included at the end of many sections an eclectic bibliography of other useful methods These bibliographies are by no means complete, but they do give some suggestions on where to go for further information.

The index contains references to substances methods authors tables and charts It is felt that a single index is easier to use than several separate indices

SECTION II

THE COLLECTION AND STORAGE OF SPECIMENS

A GENERAL CONSIDERATIONS

The handling of tissue, food, body fluids and excreta will depend upon the analyses required. Below are listed techniques that have proved useful in metabolic and field studies when ordinarily we have required hematology and analyses for nitrogen, minerals, vitamins of the B complex and ascorbic acid.

B FOOD (WHOLE DIETS IN THE FIELD)

Apparatus

1 Diet scales
2 Food chopper
3 Waring Blendor, one quart capacity
4 Suitable pots and pans
5 Two mixing cylinders of [illegible] liter capacity
6 Deep freeze unit or equivalent
7 Brown bottles with screw tops of 250 ml capacity

Reagents

1 If ascorbic acid is to be estimated, a 10% solution of oxalic acid in water is needed for stabilizing the vitamin.

Procedure

1 Weigh the total day's diet including fluid.
2 Sampling technique is critical in all kinds of food analysis. Obtain a truly representative collection of the diet. Mix this well by hand or in a mechanical grinder.
3 Weigh accurately on diet scales a sample of about 500 grams.
4 Mix this with approximately 1 liter of water. (If vitamin C is to be estimated use 200 ml of 10% oxalic acid and 800 ml of water.)
5 Homogenize the mixture in the Waring Blendor.
6 Dilute the homogenate to exactly 2000 ml.
7 Mix well by pouring from one cylinder to another.
8 Fill a 250 ml brown bottle about ¾ full and store in deep freeze.

Calculation

$$\text{gm nutrient/day} = (\text{mg nutrient/ml final mixture}) \times \frac{2000}{1000} \times \frac{\text{gm total daily diet}}{\text{gm sample used}}$$

Example

Total day s diet (including fluid) weighed 2750 gm
Sample taken for mixing was 500 gm
Final volume of mixture was 2000 ml
The final mixture contained 2 0 mg N/ml

Therefore

$$gm\ N/day = (2\ 0) \times \frac{2000}{1000} \times \frac{2750}{500} = 22\ 0$$

Precautions

1 Sampling of the diet is critical especially when whole chunks of meat are used
2 Complete homogeneity of the mixture is essential For the highest accuracy it must be possible to pipette the final mixture
3 In deep freezing bottles will break if they are too full Two thirds is about right
4 Computations are made easier if the total day s diet can be mixed for analysis not just a sample of it

C WHOLE BLOOD AND SERUM (IN THE FIELD)

A properly trained crew using convenient apparatus and facilities can easily draw 70 specimens of blood in one hour

Apparatus (assuming 100 subjects)

For drawing blood

1 Convenient table at least six feet long and comfortable chair for subject
2 Kit containing 20 ml syringes 15 ml centrifuge tubes sharp No 20 needles sponges alcohol 80% by volume and bandages
3 Racks containing ¼ oz vials prepared with heparin as described below
4 Dishpan containing clean water
5 Small pan containing isotonic saline solution made as described below
6 Autoclave or other field sterilizer

For preparing and storing serum

1 Centrifuge. Clay Adams Senior Clinical Angle Head Eight Place AC DC Centrifuges are thoroughly satisfactory
2 Wooden applicators
3 Two stainless steel 6 inch No 20 needles for transferring samples
4 Racks of ¼ oz vials for storing samples
5 At least a dozen 10 ml syringes

Reagents

1 Saline solution About 2 liters for every 100 subjects It is most conveniently prepared from commercial salt tablets designed for the preparation of physiological saline solution The usual tablet weighs 1 0 gm and should be dissolved in 113 ml of water
2 Heparin Potent preparations of the Connaught Laboratory are the most satisfactory There should be solutions of two different strengths The first is used when it is desired to keep 10 or more ml of blood from clotting The heparin is dissolved in hot physiological saline solution in such a strength that 1 drop will hold 10 ml of blood for 24 hours The second or weak strength solution is designed to be dried in ¼ oz vials to hold 2 ml of whole blood for 24 hours It is prepared in plain water in such a strength that 1 drop will hold 2 ml of blood When 100 subjects are being examined ¼ oz vials are placed in their racks one or two days before the test and one drop of weak heparin is placed in each vial and is allowed to dry

The tops are screwed on and adhes ve tape labels are attached The solut ons of heparin once made are stable in cool places for at least 2 months When dried in vials the heparin keeps indefinitely As an example of making up heparin solutions a recent batch of Connaught Laboratory heparin was shipped in vials each containing 100 mg For strong heparin 100 mg were dissolved in 14 ml of physiological saline and for weak heparin 100 mg were dissolved in 70 ml of distilled water It is obvious that these amounts vary from batch to batch.

3 Alcohol 80% for sterilız ng

Procedure

1 *Drawing blood*

a On the night before needles are sharpened and sterilized syringes are checked for chips and vials are labelled

b The six foot table is arranged so that the subject sits on a chair at one end with his right arm straightened over the table conveniently for one man to draw blood from an antecubital vein Racks containing centrifuge tubes and heparinized vials are placed near the subject s arm. Dishes containing sponges in alcohol are placed conveniently within reach of the man drawing blood A roll of 50 needles that have been carefully wrapped in gauze and sterilized is opened with just the first needle showing As needles are needed the roll is gradually opened so as not to con taminate all of them At the other end of the table are placed a dishpan contain ing clean water and the pan containing physiological saline solution The space in the mi ldle of the table is reserved for syringes

c A crew of 4 men engages in these activities The subjects are commanded to line up s ngle file Each man comes up in turn sits in the chair and straightens out his arm over the table One man behind the chair applies venous stasis by hand and another man commences to draw blood. A third man on the side op posite the chair labels a centrifuge tube and a vial with the subject s identifica tion When about 15 ml of blood have been drawn the needle is removed from the arm a sponge moistened with alcohol is placed in the antecubital space and the subject is released with instructions to maintain firm pressure by hand for 5 minutes In our experience the mere flexing of the elbow in some instances is not enough to prevent hematomas

d The man who has labeled the vials and tubes holds the vial in such a position that the operator can easily introduce about 2 ml of blood into the heparinized vial and then screws on the top and mixes the blood and heparin thoroughly by inversion DO NOT SHAKE At the same time the operator introduces the rest of the blood into the 15 ml centrifuge tube down the side gently in order to prevent hemolysis

e The fourth member of the crew washes dirty syringes and needles by receiving them from the operator He is stationed at the far end of the table A sterile needle is fitted on a clean syringe and the operator begins to draw blood from a new s bject who is be ng prepared by the second member of the crew

f After the syringe and needle are washed in clean water the needle is placed in the basin of alcohol and the syringe is prepared for use by 2 washings of about 5 ml each with physiological saline solution The residual sal ne solution in the syringe is in large part removed by vigorous pumping of the barrel The amount of saline now remaining has no significant effect on 15 ml of blood. The clean syringe is placed on the table within convenient reach of the operator

2 *Preparation of serum*

a. The centrifuge tube containing blood is allowed to stand for at least 15 min utes so that clotting is well advanced It may stand up to 2 hours without harm

b A clean wooden applicator is inserted carefully down the side of the tube to the bottom The applicator is moved around the whole circumference of the tube touching the side for the full length of the tube at all times This step breaks strands of fibrin and is essential for obtaining a good yield of serum
c The centrifuge tube is placed in the centrifuge and is centrifuged at high speed for about 10 minutes
d Just before step c vials are labeled and numbered to correspond with the samples in the centrifuge It has proved unsatisfactory to label many vials at once owing to mix ups in transferring samples of serum when this is done
e With the centrifuge tube held in the left hand and a 10 ml syringe fitted with a long needle in the right, serum is removed and is placed in a vial
f Samples of whole blood and serum in carrying cases should be stored in a convenient cool place

Precautions

1 *In drawing blood*

a The man washing syringes must be sure that needles do not become plugged that chipped syringes are removed from circulation and especially that there is no excess saline solution left in syringes that he has cleaned
b Before beginning to draw blood it is advisable to brief the whole group of subjects on the desirability of maintaining firm pressure after blood has been drawn
c Be sure that heparinized tubes are completely dry before use

2 *In preparing serum*

a Hemolysis is best prevented by making sure that centrifuge tubes are dry, that the needle is removed from the syringe before blood is introduced into the centrifuge tube and that blood is introduced gently down the side
b Make quite sure that there is adequate padding in the bottoms of the brass centrifuge cups and all centrifuge tubes to be used fit loosely in the cups without their lips touching the brass If this is not done there will be annoying breakage

D URINE (IN THE FIELD)

When a hundred or more subjects are to be studied simultaneously, the work is facilitated by the use of disposable vessels convenient storage bottles and oxalic acid as a stabilizing agent

Apparatus

1 About 120 unwaxed cardboard drinking cups (*These must be tested for substances interfering with subsequent estimations* Dixie cups No 2108 8 oz are good.)
2 Measuring cylinders of 250 ml capacity
3 Carrying racks containing 1 oz round short wide mouth amber bottles with plastic screw tops Each new batch of tops must be tested for substances interfering with estimations
4 Porcelain spatula for delivering approximately 100 mg of oxalic acid into storage bottles

Reagents

1 Oxalic acid C P powdered

Procedure

1 On the preceding night add to each storage bottle *approximately 100 mg of* oxalic acid by means of a calibrated small spatula The functions of the oxalic acid are (a) to stabilize ascorbic acid and (b) to bring the acidity of the

specimen to pH 3 to 5 which is the range of maximum stability of thiamine riboflavin and N¹ methylnicotinamide

The night before labels of adhesive tape are placed on the tops of the storage bottles and the notebooks are lined up

3 It is usual in our field surveys to collect fasting specimens in the morning The subjects are aroused at 4 30 A M and are lined up at a latrine just about 5 00 A.M At command they empty their bladders into the latrine They are then ordered to drink at least one half pint of water in order to insure diuresis They are also ordered to urinate in the next hour and a half only at command. Light activity is allowed and we customarily draw blood during this time as described in C above

4 Just before 6 30 A M each subject is issued a paper cup with his identification on it At the command all subjects urinate into their cups and are instructed to empty their bladders completely using more than *one cup if necessary After* urination the subjects place their cups in order in a convenient place *for* subsequent measuring

5 The time at the beginning and end of collection is recorded Subjects unable to urinate at command must be detained until they can urinate and the times for such men have to be noted particularly carefully

6 *It is convenient to have a crew of 3 men for measuring and storing the urine* The first man measures the volume of each specimen *reports it to the recorder* and pours an aliquot of about 25 ml into the brown urine bottle *pouring excess* urine into a waste jar and throwing paper cups into a garbage can The second man records the volumes in a notebook The third member of the crew is responsible for putting identification numbers on the 1 oz bottles to correspond with the sample from the cup screwing on tops and putting the specimens in order in a rack

7 The racks of specimens are placed in *order in their carrying boxes* and are stored *in any convenient cool place*

Calculations

1 It is desirable to record urinary excretions of vitamins in terms of milligrams *or micrograms* excreted per hour One column in the notebook should therefore show ml of urine excreted per hour calculated as follows

$$\text{ml urine per hour} = \frac{\text{ml urine collected in total period}}{\text{time in hrs and decimal fraction of hr}}$$

Example

Bladder emptied 5 05 and urine collected until 6 6

ml collected 110

Therefore

$$\text{ml urine/hr} = \frac{110}{1^{21}/_{60}} = \frac{110}{1\ 3} = 81$$

The calculations are facilitated by the line chart (Fig 1)

Precautions

1 *Always have spare cups on hand in case a few men have a large diuresis.*

2 Never put oxalic acid in the paper cups because it quickly makes the bottoms leak

3 Pouring from the cups is facilitated by pinching the cup and making it into a spout.

4 In *our experience* trying to put tops on the cups is more bother than it is worth

5 If calcium is to be measured *the urine samples must be preserved* with a *layer* of toluene Oxalic acid will precipitate out calcium in the urine

VOLUME OF URINE PER HOUR FROM TOTAL VOLUME AND TIME OF COLLECTION

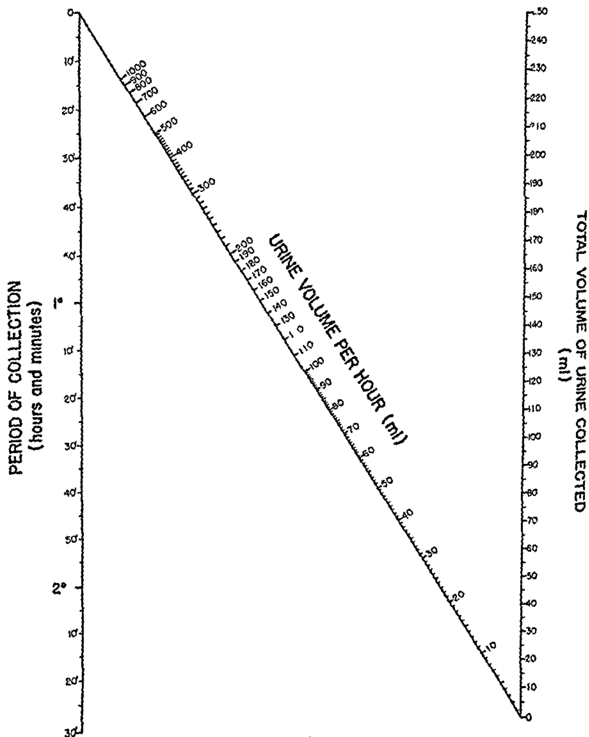


Fig 1

E TISSUE

Apparatus

1 Torsion balance (Roller Smith 25 gm capacity)
2 Mortar and pestl
3 Deep freeze unit
4 A variety of pipettes
5 A number of 15 ml round bottom centrifuge tubes
6 A number of one ounce brown bottles
7 A number of tissue grinders (Potter V R and Elvehjem C A. A Modified Method for the Study of Tissue Oxidation J Biol Chem 114 495 504 June 1936)

(The apparatus consists of a Pyrex tube of 30 ml capacity graduated to 25 ml and fitted with a revolving glass pestle that has a 15 cm glass shaft on its up p r end The shaft fits by means of a rubber stopper to the shaft of a 1/50 h p electric motor that has a built in reducing gear of 8 ½ ratio and a suitable rheo stat)

8 Centrifuge

Reagents

1 If ascorbic acid is to be estimated a 5% solution of metaphosphoric acid in water or a 10% stannous chloride solution made up in 5% metaphosphoric acid is needed for stabilizing the vitamin.
2 Carbon dioxide solid (when unstable compounds are involved)
3 Trichloracetic acid a 4% solution in water is the most generally applicable deproteinizing agent

Procedure

1 For specific analyses refer to the full details of the method as presented in the appropriate sections
2 As a general tool for preparing solutions for routine analysis of water soluble compounds such as lactic acid the tissue homogenizer has been of considerable use
   a Weigh tissue on torsion balance and drop into the bottom of the homogenizer tube (If an unstable system such as the glycolytic system of muscle is in volved the tube should be in dry ice for quick freezing)
   b Add approximately 10 ml of the appropriate deproteinizing solution. (For unstable systems this should be near the freezing point)
   c Insert the rotor of the homogenizer and grind the tissue until no discrete particles can be seen in the tube
   d Remove the rotor washing it down into the tube with a stream of deproteiniz ing solution delivered from a pipette
   e Dilute the contents of the tube exactly to the des red volume with deproteiniz ing fluid.
   f Centrifuge 10 minutes at moderate speed
   g Use aliquots of the supernatant fluid for analysis
3 Under many circumstances samples of tissue can be weighed placed in a closed container which will prevent evaporation and stored in the deep freeze until required for analysis.
4 For some estimations the tissue homogenizer is unsuitable and a mortar and p stle has to be used. Skin and bones cannot be ground in the tissue homogenizer

Calculation

See specific methods

Precautions

See specific methods.

F FECES

Apparatus

1 A deep freeze unit
2 A refrigerator
3 A Waring Blendor one quart capacity
4 A 2000 ml graduated cylinder
5 Large open mouth jars of 3 to 4 liter capacity
6 A number of 2 ml and 5 ml calibrated Yale BD *Lok Syringe (These are recommended because of their wide bore)*
7 A number of No 14 needles to fit the *above syringes (These needles should be sawed off at one inch)*

Reagents

1 Carmine *in gelatin capsules containing 600 mg each*
2 Caprylic alcohol C P

Procedure—Preparation of Subject

1 *At the time to start the collection period usually of 3 days in this laboratory,* give the subject one carmine pill by mouth Seventy two hours later give him *another carmine pill by mouth*
2 He should collect his feces as described below in a large jar until carmine first appears *in a stool Then he should collect all stools thereafter in a separate* wide mouth jar until the second dose of carmine first appears This marks the end of the 3 day collection

Procedure—Preparation of Specimen

1 The sample is collected into a large jar with a wide mouth Up to three days the sample is always kept in a refrigerator
2 At the end of that time approximately 1500 ml of water is added and the lid of the jar stoppered tightly
3 Add 1 ml of caprylic alcohol to prevent foam and shake vigorously
4 Pour portions gradually into a Waring Blendor and homogenize completely
5 Add all the homogenized portions to a 2000 ml graduated cylinder and dilute exactly to 2000 ml
6 Pour all into a large bottle or flask mix by shaking and pour aliquots into two 250 ml brown bottles Stopper well
7 Deep freeze until used for analyses
8 For individual analyses refer to the *appropriate section*
9 For almost all kinds of analyses aliquots may be measured accurately *from a Yale* BD Lok Syringe which delivers accurately to 0 1 ml *if properly handled*

Calculations

If the collection period was three days gm substance per day = (gm per ml final mixture) $\times \frac{2000}{3}$

Precautions

1 Fill bottles only 2/3 full as in freezing the expansion will cause the bottles to crack if full
2 Step 5 is critical Unless the specimen is homogenized well enough to be pipetted accurately, results will be unreliable *No discrete particles must remain in the final mixture*
3 Some subjects produce feces the color of which masks carmine *In such cases charcoal or malachite green should be substituted for carmine*

SECTION III

INSTRUMENTATION

A GENERAL PRINCIPLES OF SPECTROPHOTOMETRY

1 Transmission

The ratio of the intensity of the exit beam to that of the incident beam is called the transmission of the tube

$$\text{Transmission} = \frac{B}{A} \qquad (1)$$

Transmission is an expression of the total effect of the intercepting transparent body on the light beam including the reflection losses as well as the actual absorption of the body itself Thus transmission is a proper factor for expressing the performance of a light filter since it is a measure of its total performance It is not a term ordinarily used in spectrochemistry because the companion expression transmittance is much more convenient Both transmission and transmittance are mentioned here in order that they may be recognized as different measurements and not regarded as different terms for the same measurement

2 Transmittance

Suppose now that a test tube of water is further modified by the addition of a portion C of a soluble colorant The intensity of the exit beam will of course fall from the original value to some lower value The ratio of the intensity of this exit beam with C added to that with C' absent is called the transmittance (T) of C '

$$T_C = \frac{B}{B'} \qquad ( )$$

It is very important to notice that T_C is an expression dependent only on the nature and amount of the added constituent independent of reflection losses and of absorption losses from all other constituents of the solution

In spectrochemical procedures this independence of measurements is achieved by the use of optically identical cuvettes One of these is filled with a solution that is either free from or else contains a known proportion of the considered constituent This cuvette of solution is called the reference Another of the identical cuvettes is filled with the solution under test containing an unknown proportion of constituent but otherwise optically identical with the reference This latter is termed the sample ' The spectrophotometer is used to determine the difference in concentration C ' between the

reference and sample by measuring the relative intensities of the exit beam first with the reference and then with the sample in position

It should now be clearly understood that we are always dealing with the transmittance of the difference in concentration of a constituent between reference and sample and not with absolute concentration It will be evident also that transmittance measurements are based on the premise of optical identity and this fact must be given due consideration in the selection and handling of cuvettes and in deciding on the proper reference solution

3 Selecting the Proper Reference Solution

In clinical chemistry the reference solution will usually consist of a blank identical in composition with the sample except for the concentration of the considered constituent In some instances distilled water may prove an acceptable blank but its use should be the exception rather than the rule because the possibility of introducing light absorbing constituents with reagents is usually very real and the slight gain in convenience seems hardly worth the cost It is certain that a reference consisting of all ingredients possibly including a known proportion of the considered constituents is by far the safest selection and as explained above it is perfectly permissible to regard the considered constituent C as the difference in concentration between reference and sample The practice of employing an air reference is to be condemned

In the development of new procedures as well as in the routine applications of existing methods too much stress cannot be placed on the very obvious but frequently overlooked fact that no spectrochemical analysis can be more reliable than its reference

4 Color and Wave Length

Referring again to a cuvette of colorant the transmittance (T) of this solution will be less than that of pure water because it allows less light to pass But let us assume further that the colorant is hemoglobin Why does the solution appear red when viewed by white light?

White is actually a mixture of light rays of many different colors and only when these colors are combined in the proper proportions does the light appear white Thus when this cuvette of solution is viewed against white light it appears red because the hemoglobin has the peculiar ability to stop the blue and green light rays much more effectively than the red The exit beam thus contains a much greater proportion of red light and so appears colored red This property of a substance to interfere unequally with the passage of different colored light is termed selective absorption

It will now be apparent that because of selective absorption the T of a colored solution will depend on the color of the light used for the measurement and that the presence of a constituent will be most evident when the T measurement is made with light of that color most strongly absorbed by the constituent This then is the outstanding advantage of the spectrophotometer as compared to a colorimeter that color most strongly absorbed by the con

stituent being measured can be readily determined and the sensitivity and re liability of the analysis greatly improved by then operating at this wave length the relative effect of interfering constituents decreasing accordingly

With a filter photometer or colorimeter it is customary to express the color of the light used for the T measurement by a name such as 'blue green ' or by an arbitrary number designating the type of filter used to produce the colored light But such terms are only approximate color expressions and for absorption spectrochemistry it is both more convenient and more exact to express the color of the light in terms of wave length (λ) This numerical expres sion of color is particularly convenient because it makes possible a graphical expression of the relationship between λS and T and this spectral transmittance (S-T) curve strikingly indicates that particular wave length where the con sidered constituent absorbs most strongly and can most readily be measured

The wave length scale of most spectrophotometers is calibrated in milli microns (1 millimicron = 10 Ångstrom units = 10^{-6} millimeter) and the wave length of the measuring light is controlled by adjusting the λ knob of the in strument until the required wave length appears at the index of the λ dial The beginner should acquaint himself with the relationship between color and wave length by placing a white paper in the instrument s cuvette well and then noting the color of the light projected on this paper with various settings of the λ dial

5 Preparation of Spectral Transmittance Curves

The development of the spectral transmittance (S-T) curve of the con sidered ingredient should be one of the first steps in the rational development of any new spectrochemical method Obviously all such measurements should be made in an environment identical with that contemplated for the analysis and it need hardly be added that the final method must incorporate control measures for pH or other environmental factors that can affect the color char acteristics of the considered constituent or of any other constituents of the solution on which the spectrophotometric measurements will depend Usually the initial study will contemplate also the S T curves of possible interfering constituents and with such a group of curves before him the analyst can then intelligently select that wave length where the proposed method will be most sensitive to the concentration of the constituent being measured and least sensitive to interference

The S T curve is also of great importance in deciding the optimum wave length because if the photometric measurements are made *with monochro matic* light having the same wave length as the characteristic absorption band of the considered constituent the data will almost invariably obey the Lambert Beer Law with precision Indeed it is only when measurements are made with such monochromatic light that this law may be expected to hold exactly and while exact observance is not imperative to exact analysis it cer tainly is both convenient and reassuring With properly developed and ap plied S-T curves it will be found possible in almost every instance to select

a proper wave length and so regulate the chemistry of the analytical method that the Lambert Beer Law applies well within clinical precision.

There do occur instances when it may prove desirable to work at some wave length other than that at which the considered constituent has maximum absorption and here again the S T curve is a chart from which one may reach logical conclusions concerning the optimum wave length An example of this sort is the spectrochemical measurements of pH using the dye methyl orange If the beginner is not already familiar with the S T curves of the acid and basic forms of this indicator it is suggested that he prepare them using the same concentration in each instance Then note that the wave length of maximum pH sensitivity does not coincide with either point of maximum absorption Because the preparation of S T curves is so simple and their significance so fundamental and applicable every operator should familiarize himself with their use by examining several typical colored solutions preparing their S T curves and then deciding the wave length best suited for the spectrochemical measurement of the colored constituent in each instance

The S-T curve is prepared by measuring the transmittance of a single sample at a series of wave lengths within the range of the spectrophotometer, using a suitable reference The data are then plotted on cross section paper conveniently with transmittance (T) as ordinate and expressed as percent and with wave length (λ) as abscissa and expressed as millimicrons (mμ) Usually the points first plotted will disclose regions of low T values and additional points will then be located in these regions until the minima are established to the desired exactitude Most spectrophotometers are capable in most instances of indicating a minimum with an accuracy of 1 or 2 mμ, simply by taking a series of closely spaced T readings in the region of the critical value

The principal purpose of the S T curve is to indicate that wave length at which the considered constituent has minimum transmittance and the exactness of this indication is highly dependent on the amount of the constituent that the measuring beam is caused to penetrate If the sample solution is too dilute or the cuvette too small the amount of constituent intercepting the light beam may be insufficient to produce an exactly measurable response Conversely if the sample is too concentrated or the cuvette too wide the S-T curve may be so flattened at the region of minimum transmittance that the optimum wave length cannot be exactly ascertained It is important to remember that the curve will be most indicative when the sample concentration and depth are so adjusted that the minimum T value will fall between 5 to 90 per cent Thus good practice demands that the first step in the preparation of the S T curve be the selection of a suitable cuvette size and solution concentration

This brief discussion of S T curves is presented primarily because their significance is too little recognized or at *least too little utilized* *Actually* one of the outstanding advantages of the spectrophotometer is that it allows the development of these curves and thereby a simple and intelligent approach to the development of new spectrochemical methods and a more exact application of old ones

6 The Lambert-Beer Law

The absorption of light by a substance is due to the transmission of the individual atoms or molecules from one energy state to another The spectro photometer is an instrument capable of measuring the intensity of light ab sorbed by a given substance at a given wave length making possible identifica tion and quantitative analysis of substances in solution

The most satisfactory operation of spectrophotometers is with systems which obey Beer's Law dealing with light absorption. The law can be stated thus (a) all layers of a solution each of a given thickness absorb the same fraction of light incident upon them and (b) all layers of solution each con taining a given number of molecules absorb the same fraction of light in cident upon them Thus the intensity of light absorbed depends both on con centration of solute and length of light path through the solution

If I is intensity of incident light
dI is intensity of light absorbed
dc is concentration in moles per liter and
dl is the thickness of a layer

$$\frac{dI}{I} \propto dl \text{ and } \frac{dI}{I} \propto dc$$

$$\int_{I}^{I} \frac{dI}{I} \quad \propto \quad \int_{o}^{l} dl \qquad \text{and} \qquad \int_{I}^{I} \frac{dI}{I} \quad \propto \quad \int_{o}^{c} dc$$

$$\ln \frac{Io}{I} \propto \ l \qquad \text{and } \ln \frac{Io}{I} \propto \quad c$$

combining

$$\ln \frac{Io}{I} \propto \ lc \qquad \text{or} \qquad \ln \frac{Io}{I} = klc$$

$$\text{or } \log \frac{Io}{I} = \epsilon\, l \qquad \text{or } D = \ \ lc \qquad \text{(Equation 1)}$$

where D = optical density or $\log \frac{Io}{I}$

= molar extinction coefficient and
c = concentration in moles per liter

In this way using the last equation if D is determined experimentally for a solution of known concentration and lightpath at a given wave length the ϵ can be calculated Since ϵ is independent of c and l when Beer s Law holds c for any other solution can be calculated from density readings To test the applicability of Beer s Law optical densities of solutions of the same thickness (l) are plotted against concentration Since $\epsilon\, l = \frac{D}{c}$, $\epsilon\, l$ should be the slope of the graph which should be a straight line

If it is desired to use transmittance instead of optical density Equation 1 transforms into the following expression

$$\epsilon = K \log 1/T \qquad \text{(Equation 2)}$$

or more simply

$$\epsilon = -K \log T \qquad \text{(Equation 3)}$$

In words Equation 3 states that the concentration of solute is proportional by a negative constant to the log of the transmittance

7 The Concentration Transmittance Calibration Graph

The importance of the Lambert Beer Law is stressed here first because it is the most simple and second because it is the only rational means for translating photometer readings to expressions of the corresponding concentration of the sample The law also frequently allows the simultaneous determination of two or even three different constituents in the same sample without mutual interference

The relationship between C (Concentration) and T (Transmittance) is exactly expressed by the Lambert Beer Law in practically every instance where the requirements of this law are fully met These four requirements are

a The T measurement must be made with monochromatic light
b The T measurement must be made at a wave length corresponding to a region of the constituent's S T curves where T is substantially constant i e at the middle of a region of maximum absorption or on a 'flat portion of the curve The measurement should not be made on the ' side' of an absorption band except under unusual circumstances
c The reference must be so selected that C = 0 when T = 100%
d The nature of the sample solution must be such that its T responds only to changes in C

Equation 3 significantly states that any concentration C of a given solution is proportional to the log of its corresponding transmittance T This means that the concentration transmittance (C T) graph of this relationship will be a straight line if plotted on semi log coordinates and such a C T graph constitutes a calibration by which the concentration corresponding to any particular T value may be conveniently determined

The equation further states that this specified straight line will always intersect the point (C = 0, T = 100%) Accordingly it is possible to prepare the C T calibration graph of an analytical method without calculations To do this determine the T of one solution of known concentration C plot this known point on semi log coordinates and then draw a straight line intersecting this point and the point (C = 0 T = 100%) This line is the C T calibration graph of the method

The precision of this method of plotting will depend on how faithfully the analytical method follows the Lambert Beer Law and it is always advisable to prepare and measure the T of several solutions of known concentration within the contemplated range of the method When plotted these several points serve both to confirm the validity of the law and to discover erroneous or careless technique The points should fall on a straight line

Let it be repeated the technique of almost any determination can be so developed that the data follow the Lambert Beer Law with precision provided

that measurements are made with a true spectrophotometer When these con ditions are met the construction of the corresponding C T calibration graph is fully as simple as it sounds

8 Optical Density

It is sometimes convenient in spectrochemistry to express the light stop ping ability of a sample in terms of its optical density D when

$$D = 2 - \log \text{percentage transmittance}$$

The advantage of this method of expression is that D values are directly proportional to corresponding C values For example

The optical density of a solution (let us say copper carbamate that has been extracted from serum) is designated as D ml the serum blank as Ds and the reagent blank as Dr Copper concentration is obtained by the following calculation

a K is d termined from the calibration curve
b D ml − (Ds + Dr) = Dcu
c Dcu × K × 100 = Copper micrograms per 100 ml

B SPECTROPHOTOMETER COLEMAN JUNIOR MODEL 6

1 General Principles of the Spectrophotometer

a. Spectrophotometer

A spectrophotometer as its name implies is really two instruments in a single case a spectrometer and a photometer A spectrometer is a device for producing colored light of any selected color and when used as a part of a spectrophotometer such a unit is usually called a monochromator and is usually calibrated to express the color of the monochromatic light it produces in terms of wave length

A photometer is a devi e for measuring the inten ity of l ght and when combined in a spectrophotometer is used to measure the intensity of the monochromatic beam pro duced by the associated monochromator Usually the photometric measurement is made both with a reference and a control sample interposed in the l ght beam the difference between the two intensity measurements then be ng a measure of the trans mittance of the sampl at the wave length of the te t

b The Monochromator

The monochromator of the Junior Clinical Spectrophotometer is illuminated by a 6 500 exciter lamp Th s lamp is powered by a 6 volt storage battery (or suitable transformer) and is turned on and off by a switch mounted integral with the fine control knob of the instrument The filament of this lamp is a thin straight coil about 0 3 inches long and exactly positioned with respect to the prefocus base of the lamp This base registers exactly in a special lamp socket mounted on the in strument s lamp arm so that the filament thus occupies a very exact position in the monochromator optical system It is very impo tant to not that exc ter lamps must accordingly be very carefully handled to avoid possible bending of the prefo us base and the calibration of the instrument verified each time the exciter lamp is disturbed.

Light from the exciter lamp filament enters the double lens system of the mono- chromator and were this an ordinary opt cal system would be caused to form a sharp white image at the plane of the monochromator exit slit However there is inter posed between the two condens ng lenses a diffraction grating and this grating has the

peculiar ability to bend the transmitted light, the amount of the bending b ing pro portional to the wave length As a result of this grating the anticipated white ima,e is actually "smeared and there appears instead at the plane of the exit slit a true and brilliant spectrum Most of this spectral image falls on one or the other of the slit sidewalls and is stopped that narrow portion of the spectrum falling directly on the slit is not intercepted and so emerges alone from the opposite side of the slit as a monochromatic beam

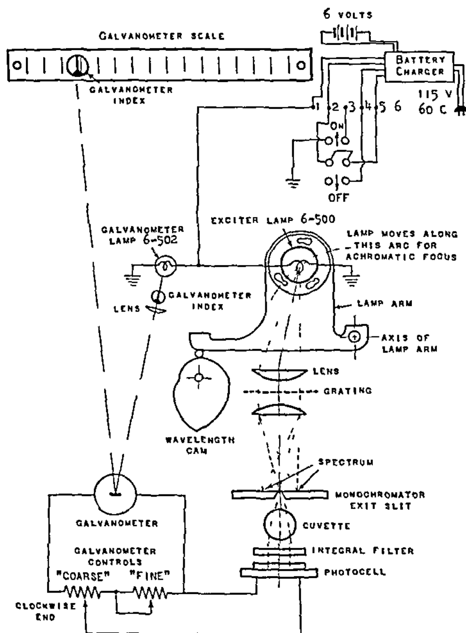


Fig * — The Coleman Junior Spectrophotometer Model 6A (R produc d by permission of the manufacturers)

As indicated the position of the lamp filament may be altered by moving the lamp arm under compulsion of the wave length cam and this cam is rigidly fixed to the wave length dial Thus, as the calibrated λ dial is rotated the lamp filament is swung across the optical axis of the monochromator resulting in a corresponding shift of the spectral image The design of the in trument is such that that portion of spectral light actually passing through the slit always has exactly that wave length value con currently indicated on th λ dial i e light of the desire l wave length is delivered from the exit slit of the monochromator simply by adju ting the instrument s λ dial to read that wave length The position and curvature of the arc through which the filament moves is such that the waveband pa sing the slit is always in sharp focus

c The Photometer

The photometer of this instrument consists of a barrier layer photocell connected by a suitable control circuit to a projection galvanometer illuminated by a 6 50? gal



SCHEMATIC DIAGRAM OF JUNIOR CLINICAL SPECTROPHOTOMETER OPTICAL AND ELECTRICAL SYSTEMS

Fig 3

vanometer lamp and projecting its index image on a galvanometer scale mounted in the top of the instrument case Mounted integral with the photocell is a correction filter selected largely to compensate for the very great differences in the photocell response to light of different wave lengths It is to be understood that this compensation is by no means complete and that when the λ dial is turned the position of the index may change markedly The method of operation of the galvanometer controls is evident from the circuit diagram (Figs 2 and 3)

It will be noted that the galvanometer of the Junior Clinical spectrophotometer is double calibrated with a linear transmittance (T) scale printed in black and with a logarithmic density (D) scale appearing in red.

d. The Optimum Transmittance Range

Errors in position of the galvanometer index due to instrument limitations and errors of the operator in reading the galvanometer scale together constitute the absolute instrument error a value entirely independent of the nature of the analytical method and of the manipulative skill of the operator The effect of this absolute error on the precision of an analysis is called the effective instrument error

The magnitude of the effective error corresponding to a given absolute value is surprisingly dependent on the transmittance of the sample being tested and as a consequence the efficacy of an analytical procedure may well depend quite as much on the dilution ratio selected as on the verity of the chemical reactions involved This is a fact the analyst too frequently disregards in the development of his new procedures although its observance is always well repaid by more exact and consistent data

2 Setting Up and Calibration of the Instrument

a. Mounting and Connecting

(1) Place the instrument on a reasonably level surface free from extreme vibration mounting one round sponge rubber pad under each instrument foot to minimize any slight vibrational effects that might otherwise disturb exact galvanometer readings

(2) Connect the twisted black and red pair of wires to either a 6 volt storage battery or to a constant voltage transformer delivering not less than 25 watts at 6 volts The polarity of this connection is immaterial

(3) If a storage battery is used always connect its positive (+ red) terminal to the positive (+ red) lead from the battery charger and connect its negative (– black or unmarked) terminal to the negative (– colorless) charger lead Connect the line cord plug of the battery charger to the power source indicated on the charger instrument plate Do not attempt to charge the battery during a test because variations of charger potential with line voltage changes will cause unstable readings

(4) Preferably locate the instrument in a position where strong light cannot fall on its panel or into the cuvette well If this condition cannot be met then always place the 6 312 light shield over the cuvette well during every reading

b Adjusting the Mechanical Galvanometer Zero

The Junior is equipped with a very sensitive but very rugged taut suspension galvanometer The zero adjustment for this element is located under the raised housing just to the left of the cuvette well. If the spectrophotometer is not disturbed or its leveling altered then this galvanometer adjustment remains very stable The purpose of the raised housing is to protect the galvanometer adjusting lever from any accidental dislocation

With the instrument set up and connected as specified in the preceding section first adjust the galvanometer zero as described below Always verify the galvanometer zero adjustment before undertaking an analysis and if necessary rectify It may con

ven ently be checked at any t me dur ng an analys s and this procedure is recom mended during prolonged rout ne exam nat ons where extreme prec s on is demanded.

(1) Turn the instrument on by rotat ng the fine knob clockw se until its sw tch cl cks and the λ d al s llum nated

(°) Desens tize the galvanometer c rcu t by rotat ng the coarse knob to its ex treme counterclockw se pos t on and the fine knob as far counterclockw se as s poss ble w thout turn ng off the switch

(3) Darken the photocell. If a cuvette adapter is ava lable the photocell is con ven ently darkened by plac ng the adapter in the well w th its key 90 clockwise to the keyway of the well In th s pos t on the adapter is not fully seated and also ts w ndow s not n the path of the l ght beam An alternat ve method is to place a sl p of opaque paper n the well so that the monochromator beam is completely stopped cover ng the well w th the l ght sh eld. W th the photocell darkened there is no electr cal input to the galvanometer wh ch may now be caused to read exactly zero on the black scale by moving the galvanometer ad just ng leve To ve fy the galvanometer zero perform steps 1 ° and 3

(4) W th a penc l po nt or other conven ent tool reach under the hous ng and move the galvanometer adjust ng lever unt l the galvanometer index reads exactly ze o on the black % T scale i e at the left hand end. Do not alter th s adjust ment dur ng an analys s except to correct

Verifying the Wave Length Calibration

O d nar ly th s operat on s not necessary yet confirmat on of the λ cal brat on s such a qu ck and s mple task that t may well be ncluded n the in tial setup It must be undertaken f the exc ter lamp s d sturbed A 6 400 d dym um standard filter is requ red for th s test

The λ cal brat on s ver fied as follows

(1) Turn on the nstrument and allow to warm up for 5 m nutes before proceed ng

(°) Set the wave length scale to read 610 exactly

(3) Insert the 6 400 d dym um cal brat ng fil er in the cuvette well but w th ts key at r ght angles to the cuvette well keyway W th th s angular pos t on ng the photo cell s completely darkened

4 Set the galvanometer to mechan cal ze o as descr bed n sect on b above

(5) Remove the 6 400 cal b at ng filter cover the well open ng and then adjust the coarse and fine knobs unt l the gal anometer index l ne co nc des exactly with 100% T on the black galvanometer scale (Tap the instrument f the ndex tends to st ck off s ale)

(6) Now place the p e ously cleaned 6 400 cal b at ng filter in cuvette well with its key ente ng the well keywa Make sure that the filter is now fully seated n well.

(7) Read the new pos t on of the galvanometer ndex l ne on the % T galvanometer scale If this measured value of T checks w th n + 3 s ale d v s ons of the value of T engraved on the 6400 cal brat ng filter the λ cal brat on is correct w th n ° m ll microns and sat sfacto y

If by any chan e the λ cal brat on i fo n l to have t en shifted n trans t refer to sect on (d) below for full cal b at ng deta ls

When the λ cal bration s found cor ect the galvanometer zero properly adjusted and the nstrument mounted and connected as spec fi d t s ready for operat on

d. Calibration of Instrument When the λ Calibration Is Not Correct

(1) Set the instrument on ts s de unscrew the four rubber feet and remove the sheet me al bottom to expos the lamp arm E and adjustments F and G (see F g 3)

( ) Loosen set screw F on a m F thereby f ee ng the cal brat ng screw G

vanometer lamp and projecting its index image on a galvanometer scale mounted in the top of the instrument case Mounted integral with the photocell is a correction filter selected largely to compen ate for the very great differences in the photocell response to light of different wave lengths It is to be understood that this compensation is by no means complete and that when the λ dial is turned the position of the index may change markedly The m thod of operation of the galvanometer controls is evident from the circuit diagram (Figs 2 and 3)

It will be noted that the galvanometer of the Junior Clinical spectrophotometer is double calibrated with a linear transmittance (T) scale printed in black and with a logarithmic density (D) scale appearing in red

d. The Optimum Transmittance Range

Errors in position of the galvanometer index due to instrument limitations and errors of the operator in reading the galvanometer scale together constitute the absolute instrument error a value entirely independent of the nature of the analytical method and of the manipulative skill of the operator The effect of this absolute error on the precision of an analysis is called the effective instrument error

The magnitude of the effective error corresponding to a given absolute value is sur prisingly dependent on the transmittance of the sample being tested and as a con sequence the efficacy of an analytical procedure may well depend quite as much on the dilution ratio selected as on the verity of the chemical reactions involved This is a fact the analyst too frequently disregards in the development of his new procedures although its observance is always well repaid by more exact and consistent data

2 Setting Up and Calibration of the Instrument

a Mounting and Connecting

(1) Place the instrument on a reasonably level surface free from extreme vibration mounting one round sponge rubber pad under each instrument foot to minimize any slight vibrational effe ts that might otherwise disturb exact galvanometer readings

(2) Connect the twisted black and red pair of wires to either a 6 volt storage battery or to a constant voltage transformer delivering not l ss than 25 watts at 6 volts The polarity of this connection is immaterial

(3) If a storage battery is used always connect its positive (+ red) terminal to the positive (+ red) lead from the battery charger and connect its negative (– black or unmarked) terminal to the negative (– colorless) charger lead Connect the line cord plug of the battery charger to the power source indicated on the charger instrument plate Do not attempt to charge the battery during a test because variations of charger potential with line voltage changes will cause unstable readings

(4) Preferably locate the instrument in a position where strong light cannot fall on its panel or into the cuvette well If this condition cannot be met then always place the 6 312 light shield over the cuvette well during every reading

b Adjusting the Mechanical Galvanometer Zero

The Junior is equipped with a very sensitive but very rugged taut suspension gal vanometer The zero adjustment for this element is located under the raised housing just to the left of the cuvette well If the spectrophotometer is not disturbed or its leveling altered then this galvanometer adjustment remains very stable The purpose of the raised housing is to protect the galvanometer adjusting lever from any accidental dislocation

With the in trument set up and connected as specified in the preceding section first adjust the galvanometer zero as described below Always verify the galvanometer zero adjustment before undertaking an analysis and if necessary rectify It may con

veniently be checked at any time during an analysis and this procedure is recommended during prolonged routine examinations where extreme precision is demanded.

(1) Turn the instrument on by rotating the fine knob clockwise until its switch clicks and the λ dial is illuminated

(2) Desensitize the galvanometer circuit by rotating the coarse knob to its extreme counterclockwise position and the fine knob as far counterclockwise as is possible without turning off the switch.

(3) Darken the photocell If a cuvette adapter is available the photocell is conveniently darkened by placing the adapter in the well with its key 90 clockwise to the keyway of the well In this position the adapter is not fully seated and also its window is not in the path of the light beam An alternative method is to place a slip of opaque paper in the well so that the monochromator beam is completely stopped covering the well with the light shield. With the photocell darkened there is no electrical input to the galvanometer which may now be caused to read exactly zero on the black scale by moving the galvanometer adjusting lever To verify the galvanometer zero perform steps 1 2 and 3

(4) With a pencil point or other convenient tool reach under the housing and move the galvanometer adjusting lever until the galvanometer index reads exactly zero on the black % T scale i e at the left hand end Do not alter this adjustment during an analysis except to correct

c Verifying the Wave Length Calibration

Ordinarily this operation is not necessary yet confirmation of the λ calibration is such a quick and simple task that it may well be included in the initial setup It must be undertaken if the exciter lamp is disturbed A 6 400 didymium standard filter is required for this test

The λ calibration is verified as follows

(1) Turn on the instrument and allow to warm up for 5 minutes before proceeding

(2) Set the wave length scale to read 610 exactly

(3) Insert the 6 400 didymium calibrating filter in the cuvette well but with its key at right angles to the cuvette well keyway With this angular positioning the photocell is completely darkened.

(4) Set the galvanometer to mechanical zero as described in section b above

(5) Remove the 6-400 calibrating filter cover the well opening and then adjust the coarse and fine knobs until the galvanometer index line coincides exactly with 100% T on the black galvanometer scale (Tap the instrument if the index tends to stick off scale)

(6) Now place the previously cleaned 6 400 calibrating filter in cuvette well with its key entering the well keyway Make sure that the filter is now fully seated in well

(7) Read the new position of the galvanometer index line on the % T galvanometer scale If this measured value of T checks within ± 3 scale divisions of the value of T engraved on the 6 400 calibrating filter the λ calibration is correct within 2 milli microns and satisfactory

If by any chance the λ calibration is found to have been shifted in transit refer to section (d) below for full calibrating details

When the λ calibration is found correct the galvanometer zero properly adjusted and the instrument mounted and connected as specified it is ready for operation

d. Calibration of Instrument When the λ Calibration Is Not Correct

(1) Set the instrument on its side unscrew the four rubber feet and remove the sheet metal bottom to expose the lamp arm E and adjustments F and G (see Fig 3)

(2) Loosen set screw F on arm E thereby freeing the calibrating screw G

(3) Now if the value of T measured at step (7) is less than that engraved on the calibrating filter turn the calibrating screw ' G'' clockwise about ⅛ turn for each 5 percent difference in T (or vice versa) Then lock in the new position by slightly tightening on screw F

(4) Repeat steps c (1) to c (7) If the measured and specified values *of T still fail* to agree within 3% repeat steps d (1 4)

(5) When adjustment is satisfactory tighten screw F'' firmly and then again repeat steps c (1) to c (7) as a final check

Note When replacing the sheet metal bottom make sure that its drain hole is *below the cuvette well*

3 Instructions for Routine Use of the Instrument

a Insert in the cuvette well a cuvette adapter of the proper size to accept the type of cuvette specified in the contemplated analytical method *Standard calibrated* cuvettes may be procured from the company

b Turn on the instrument by rotating the fine knob clockwise until the switch clicks and the λ dial is illuminated.

c Verify the galvanometer zero setting and readjust if necessary

d Adjust the λ dial to that wave length specified in the contemplated analytical method

e Wipe clean and then insert at the proper angle in the cuvette well a cuvette containing a sufficient volume of the reference solution (This volume will depend on the size of the cuvette)

f Adjust the coarse and fine knobs until the galvanometer index reads that value specified in the contemplated analytical method. (Usually this will be 100% T if the black transmittance scale is used or 0 if the red density scale is utilized.)

g Remove the reference cuvette Wipe clean and then insert at the proper angle a *similar cuvette containing a portion of the sample solution*

h Read the position of the galvanometer index on the ame (red or black) galvanometer scale as was used for the initial adjustment (f)

i Refer to tables curves or scale reading for the actual concentration of the sample solution as directed in the contemplated analytical method

C BECKMAN PHOTOELECTRIC QUARTZ SPECTROPHOTOMETER (MODEL DU)

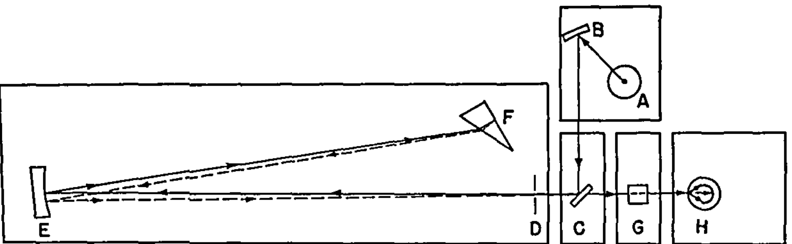
1 General Construction

a Electrical System

The Beckman Spectrophotometer is essentially a potentiometer Light passing through the quartz prism monochromator and the solution falls on a light sensitive phototube inducing in it a voltage proportional to the intensity of the light incident upon it This voltage disturbs the balanced potentiometer which must be rebalanced. *This* rebalancing is done with a variable resistance calibrated in % T and D In operation the circuit is first balanced with a blank or pure solvent in the light path then with the solution in the light path If the potentiometer reading of the solvent is arbitrarily set at zero D (100% T) then the balanced potentiometer with the solution in the l ght path will read d rectly in % T or D

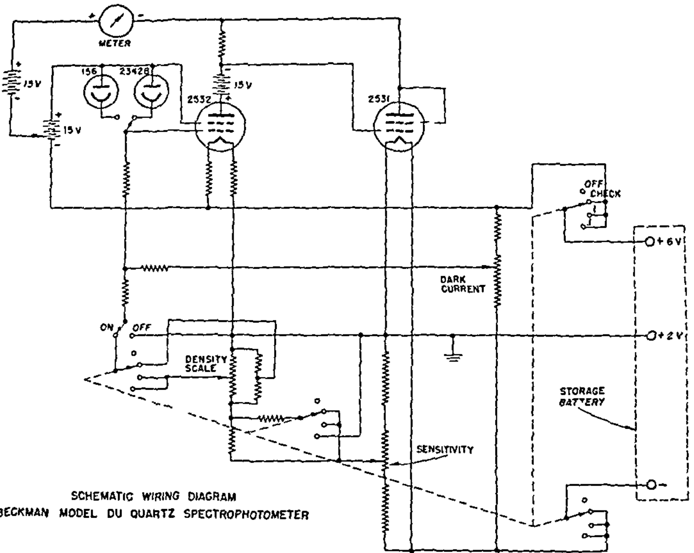
b Optical System

The optical system of the spectrophotometer is shown schematically in Figs 4 and 5 An image of the light source A is focused by the condensing mirror B and diagonal mirror C on the entrance slit at D The entrance slit is the lower of *two slits*



SCHEMATIC DIAGRAM OF OPTICAL SYSTEM

Fig 4



SCHEMATIC WIRING DIAGRAM
BECKMAN MODEL DU QUARTZ SPECTROPHOTOMETER

Fig 6

windows are available with 10 mm 25 mm and 50 mm light paths and may be used for measurements on liquids over the entire wave length range of the instrument. With the exception of the demountable cells and certain gas cells all of the above cells are of fused construction and are not cemented

g Phototubes

Two phototubes are furnished with each instrument mounted in a sealed desiccated compartment adjoining the cell compartment A sliding rod brings either tube into position and simultaneously switches their electrical connections A switch coupled to the phototube aperture shutter permits the dark current of the phototube to be checked at any time without removing cells or changing any control setting

A cesium oxide phototube with high sensitivity from 1000 millimicrons to 600 millimicrons and a blue ultraviolet sensitive phototube in ultraviolet transmitting envelope with high sensitivity from 6 5 millimicrons to 00 millimicrons are supplied with the Model DU Spectrophotometer

h Spectral Range

The wave length scale is calibrated from 200 millimicrons in the ultraviolet region through the entire visible range and in the infrared to 2000 millimicrons As no single phototube exhibits maximum sensitivity over the entire spectral range each Beckman instrument is equipped with two phototubes one having maximum sensitivity in the red infrared region and the other having maximum sensitivity in the blue ultraviolet region Either tube can be instantly switched into use

At present the short wave length limit of operation is governed by several practical considerations such as the limiting sensitivity of phototubes the intensity of radiation from the light source radiation losses at reflection surfaces and the opacity of fused silica or special glasses used in the construction of absorption cells and phototubes The upper wave length limit of operation is governed primarily by the limiting sensitivity of available photoelectric tubes Full sensitivity performance is guaranteed for all instruments to 1000 millimicrons and satisfactory operation to 1100 millimicrons may reasonably expected

i Sensitivity

The phototube and amplifier circuits of the Beckman Photoelectric Spectrophotometer are unusually stable and sensitive A useful current amplification of 5×10^7 permits full scale readings on a rugged milliammeter with as little as $2\,5 \times 10^{8}$ watts of radiant energy Because of this high sensitivity slit openings can be narrowed to secure maximum resolution Measurements can be made over most of the spectral range with nominal band widths of less than one millimicron

j Accuracy

Precision optical and mechanical parts permit slit openings and wave length settings to be reproduced with great fidelity Wave length settings are reproducible to 0 05 millimicrons in the ultraviolet and 0 5 millimicrons in the infrared Optical parts are securely mounted in a rigid casting and are not affected by vibration, dirt or fumes

Stray light often an unsuspected source of error in spectrophotometric measurements has received *special attention Instead of a replica grating the crystal* quartz prism is used. Carefully designed baffles and optical surfaces of the highest quality keep diffuse reflection at a minimum The total stray light effect over all but the extreme ends of the spectral range is less than 0 1%

For measurements between zero and 10% transmittance the transmittance scale is expanded by merely throwing a switch so that the entire transmittance scale reads 0 to 10% instead of 0 to 100%

2 Setting Up and Calibrating the Instrument

a Power Requirements

The power required for operation of the Beckman DU Quartz Spectrophotometer, other than that supplied by the dry batteries installed in the instrument is supplied from a 6 volt storage battery To insure the tube stability a battery of approximately 1[illegible]0 ampere hours rating (i e a heavy duty truck battery) is recommended.

For operation securely attach the battery clamps marked – +[illegible] and +6 to the corresponding voltage terminals of the storage battery If two batteries are used in parallel it is not necessary to parallel the 2 volt terminals This apparatus requires 6 Burgess dry cell batteries #5510 (Beckman #911) each 7½ volts

b Selector Switch

The main instrument battery switch has four positions OFF CHECK, 1 0 0 1 In the *OFF* position all battery circuits within the monochromator and those to the phototube compartment are disconnected (Separate switches are provided for the tungsten lamp and hydrogen discharge lamp) The CHECK position provides a very convenient means of adjusting the instrument for 100% transmittance on the solvent without having to set the transmittance scale to 100% because the transmission slide wire is replaced with a fixed resistor circuit calibrated to be the equivalent of 100% transmittance with the selector switch in 1 0 position Measurements on the unknown solutions are usually made with the switch on 1 0 The transmittance scale is then read 0% to 110% transmittance and the density ty scale infinity to zero The useful part of the density scale is from 0 to [illegible] 0 Position 0 1 gives a tenfold increase for sensitivity in range from 0 to 11% and is used for samples having less than 11% transmittance In this case the transmittance scale is read 0 to 11% transmittance and the density scale 1 0 to infinity with the readable calibration being 1 0 to 3 0 density units (read by adding 1 0 to the density scale reading) The 0 1 position also permits measurements with extremely narrow spectral band widths

c Phototube Selection

The Model DU is equipped with ultraviolet sensitive and red sensitive phototubes *for* the range [illegible]0 to 1000 millimicrons The proper phototubes to use is determined by the spectral range to be investigated. The red sensitive phototube required for use *above* 6 5 millimicrons (but usable to 400 millimicrons) is in position when the knob *on the* phototube housing is pushed IN The ultraviolet sensitive phototube for use *below 6 5* millimicrons is in position when the knob is pulled OUT as far as possible The two electronic tubes of the amplifier circuit are also mounted in the *phototube compartment*

d. Light Source

The *tungsten lamp is used from 3 [illegible] to 1000* millimicrons and the hydrogen discharge lamp is used from [illegible]0 to 50 millimicrons The lamp housings may be interchanged by pulling up the locking slide in the left side of the lamp housing The power *for* operation of the tungsten lamp is supplied from the storage battery through the spectrophotometer circuit A special electronic power supply unit Part No [illegible]0 is necessary to provide current for operation of the hydrogen discharge lamp

e. Filter Slide

The filter slide is located in the mounting block between the slit plate and cell compartment and is positioned by means of the knob on the front of the instrument. The front position knob pushed IN is blank and is used *for measurements* in the range 400 to 1000 millimicrons—also with the hydrogen tube The second position contains a Corning No 9863 Red Purple Corex A filter and is used in the range of 400 to [illegible]0 millimicrons with tungsten lamp The third position is blank and *may* be fitted with special filter lens or screen for various applications

f Sensitivity Control

Maximum accuracy on the percent transmittance measuring circuit is attained when the sensitivity knob is used 1 to 3 turns from its clockwise limit and requires com paratively wide slit openings Even these slit openings give a spectral band width of less than 1 millimicron over most of the spectral range of the instrument With the sensitivity knob in this position transmittance readings may be easily made to 0 1% At this high sensitivity needle fluctuations may be encountered The fluctuations may appear objectionable to the user but do not effect the accuracy to which measure ments can be made They may be reduced at some sacrifice of accuracy by operating with the sensitivity knob near its counterclockwise limit or at reduced speed of op eration but no sacrifice in accuracy by installing IR /0 348B Condenser Assembly The maximum resolution of the monochromator which may be realized with the en ergy measuring system is obtained when the sensitivity knob is near its counter clockwise limit, or when the switch position 0 1 is used because under these con ditions less light is required for balance permitting narrow slits to be used

g Characteristics and Use of Absorption Cells and Holder

The Corex cells regularly supplied with the DU Spectrophotometer are used in the wave length region from 3?0 to 1000 millimicrons Cells made of silica glass must be used in the lower ultraviolet region to 220 millimicrons Silica cells are not limited in use to the ultraviolet region but may be used through the entire spe tral range of the instrument

All absorption cells of both Corex and silica are filled with distilled water and tested for percent transmittance in the spectrophotometer This transmi sion of Corex cells must be at least 75% of that of an air light path and this transmission of silica cells must be at least 70% of that of an air light path

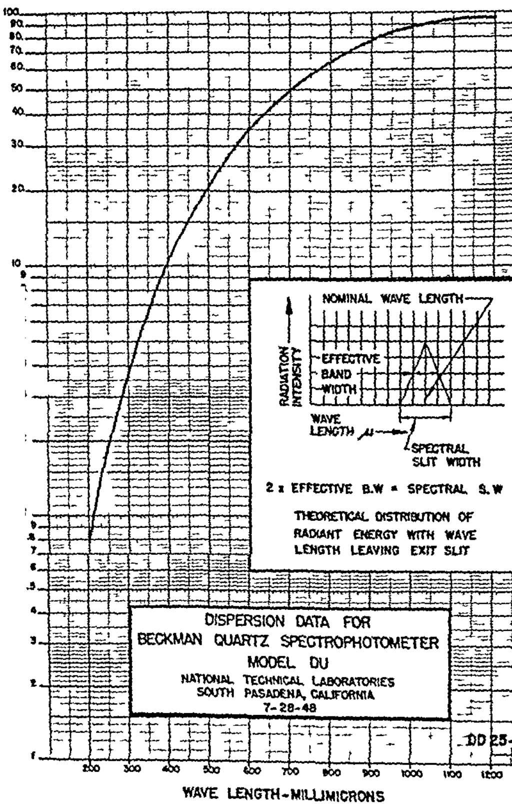
All standard absorption cells are matched for light transmission at various wave lengths. Matching is done in a spectrophotometer with the cells filled with distilled water Corex cells are matched to within 1 0% transmittance at 800 500 400 and 350 milli microns and to within 2% at 3 0 millimicrons Silica cells are matched to within ?% transmittance at 350 300 2?0 and 240 millimicrons and to within 3% at 2 0 milli microns All cells approach perfect matching at longer wave lengths

These cells are also matched with respect to the mean light path length to within 0 5% in that portion of each c ll through which the beam passes Perfect matching of the light path length is not usually necessary providing it is known Each 10 mm cell is marked giving its light path length to the nearest thousandth of a centi meter

The absorption cells should be thoroughly cleansed with distilled water or other suit able solvent before use Do not habitually use hot concentrated acids which might etch the polished surfaces Remove cells from the holder for cleaning and filling and before replacing carefully wipe the outside surfaces For the most precise work however the outside surfaces should not be wetted or touched during a series of measurements

It is recommended that the cell holder be used with the corner springs toward the phototube and that it be removed from the compartment for loading and unloading It is also recommended that one position in the holder be consistently reserved for the cell containing the solvent One of the ground glass sides of each cell should be marked and it should always be oriented the same way in its holder position.

When the holder containing filled cells is placed on the slide in the cell compartment make certain it is seated properly with knob pushed *IN* Each cell is moved into the light beam by pulling the slide knob to the correct detent stop The cell compart ment cover must be in place when measurements are being made



100
90
80
70
60
50
40
30
20
10
9
7
6
5
4
3
2
1
NOMINAL WAVE LENGTH
RADIATION INTENSITY
EFFECTIVE BAND WIDTH
WAVE LENGTH
SPECTRAL SLIT WIDTH
2 x EFFECTIVE B.W = SPECTRAL S.W
THEORETICAL DISTRIBUTION OF RADIANT ENERGY WITH WAVE LENGTH LEAVING EXIT SLIT
DISPERSION DATA FOR
BECKMAN QUARTZ SPECTROPHOTOMETER
MODEL DU
NATIONAL TECHNICAL LABORATORIES
SOUTH PASADENA, CALIFORNIA
7-28-48
DD 25-
200 300 400 500 600 700 800 900 1000 1100 1200
WAVE LENGTH-MILLIMICRONS

Fig 1.

h Nominal Band Width

Fig 6 gives the "band width versus wave length" relationship from which the required slit openings for a given spectral band width can be determined for any wave length of interest. If it is desired to make measurements with the very minimum spectral band width, the instrument switch may be used in the 0.1 position and the slide wire set to 100% transmittance for balancing with the solvent or "blank" in the light beam. The sensitivity knob should be near its counterclockwise limit. In this procedure the transmission scale is read 0 to 110%—not 0 to 11%.

For measurements at a predetermined spectral band width the necessary slit width must be calculated with the consideration that optical aberrations tend to increase that slit width by approximately 0.02 mm. If X is equal to the actual slit width to be used at one wave length corresponding to the nominal band width it may be calculated as follows

W_D = Spectral band width per 1 mm slit opening
W_S = Spectral band width to be used for measurement

$$X = \frac{W_S - 0.02\, W_D}{W_D}$$

For measurements at wide spectral band widths the light intensity to the phototube is decreased by inserting a fine screen of proper mesh size (determined experimentally) in position 3 of the filter slide.

i Effects of Band Widths on Transmission or Reflectance Measurements

The apparent percent transmission at any wave length will usually depend on the resolution or effective band width and will include such influences as the slope of the transmission curve within the wave band being transmitted and the variation of the photo receiver. It is usually easy to determine if these effects are significant by increasing or decreasing the slit width by a factor of 2 or more. It is suggested that whenever reliable values of percent transmission must be obtained for instance when intercomparing the results between instruments or when changing from the red sensitive to the ultraviolet sensitive phototube that the narrowest possible slit widths be used and the above check for the effective resolving power be made.

j Calibration of Cells

Because each set of cells is matched at the factory with respect to light transmission it is seldom necessary to apply individual cell transmission corrections. However when using shorter wave lengths or where extreme precision is necessary the cells should be calibrated. The calibration of each cell in a group of cells to be used should be made with respect to one cell selected as the reference cell. This calibration should be checked at several points in the wave length region to be used. The cells must be calibrated at each wave length of use when filled with solvent. Do not calibrate unfilled cells because variation in reflection from unwetted internal cell surfaces will alter standardization values.

Clean and fill each cell with distilled water or other solvent. Place the cells in the cell compartment and replace compartment cover. Set wave length dial at the wave length desired (see Sections c d e g). Turn the lamp ON set selector switch to 1.0 and the transmission dial to zero density. Turn shutter switch OFF, and balance meter needle with dark current knob. Turn shutter switch ON rotate sensitivity knob to within one turn of its clockwise limit and approximately balance the needle with the slit dial. Balance the needle accurately with the sensitivity knob and record the density reading. Slide each of the cells into the light beam and balance the needle with the transmission dial recording the reading as density.

Select one cell as the reference cell to contain the solvent throughout all measurements. Subtract its density reading algebraically from the values for all cells. Record these resulting values as D

Subsequent experimental density measurements D are corrected to actual sample density D by algebraically adding the correction D

Example

D =	− 003	Calibrated cell correction.
D =	1 5	Measured density of sample and cell
D =	175 − 003 = 17	Actual sample density

k. Calculation of Solution Concentration

For quantitative absorption measurements where the unknown component is the only absorbing material at the wave length of measurement it is neces ary to consider the concentration of the solution and light path length of the cell Calculations of this sort may be carried out as follows

Calculate k (extinction coefficient) at each wave length of measurement from measure ments made on a solution of known concentration as follows

$$D - k\ c\ d = \left(-\log \frac{T}{100}\right)$$

c = concentration of sample in solution
d = l ght path of th cell
D = corrected optical density
T = corrected percent transmittan e

Using the value of k concentrations of the unknown in other solutions may be calcu lated from this same formula by sub t tuti g the proper density value and l ght path length of the cell

l Focusing of the Tungsten Lamp

When a new tungsten lamp is receive l in its housing from the factory only the final focusing adjustment if any should be needed Extensive refocusing is seldom neces sary but may be carried out as follows

(1) Remove cells and holder from compartment unscrew the four knurled thumb screws on the en l of the phototube housing and remove the housing and absorp tion cell compartment Unscrew the four screws that fa ten the mounting block (containing entrance mirror and filter sl de) to the monochromator and remove the block tilting the top away from the end plate in order not to drop the filter slide Note mirro mounting and adju tment but DO NOT TOUCH ANY MIR ROR SURFACES

( ) Turn on the current and adju t the lamp and mirror screws so that the light beam comes through the hole in the front of the lamp housing Hold a piece of white paper in the l ght beam to locate the crescent shaped focal po nt which should appear oppos te the monochromator lower (entrance) w ndow with the center of the image about 5/16 out from the windo To raise or lower the l ght beam turn the upper lamp adjust ng screw If this adjustment is insufficient tilt the con densing mirror slightly

(3) The condensing mirror in the lamp housing may be extensively adju ted by means of the three small scre s at the left side the screw in the bushing rotates the mir ror from side to side the two clamping screws above and below bushing are loo ened to t lt the mirror or to sl de it toward or away from the lamp and must be tightened after adjustments are made CAUTION Do not release screws more than two or three revolutions as there is danger of releasing the mirror mounting

(4) To direct the light beam toward or away from the monochromator end plate turn the mirror adju ting screw in the bushing To change the focal point of the image turn lower lamp adjusting screw If this adjustment is insufficient, shift the whole mirror a embly toward the lamp to lengthen the focal distance or vice versa. After the focal point adju tments are mad replace filter slide and mounting block securely

(5) Set slit opening at 2 mm and wave length scale at about 800 millimicrons Push filter slide knob in Through the exit slit the light beam should appear as a magnified (red) image of the lamp filament If it does not a slight adjustment of either the condensing or entrance mirror should bring out the horizontal filament lines To adjust entrance mirror insert a screw driver into the hole in the front of the mounting block and turn the screw at the back of mirror After a good visual adjustment has been made replace the absorption cell compartment and phototube housing

(6) Pull phototube knob out Push filter slide knob in Turn the instrument switch to "CHECK," the shutter switch to 'OFF' and then adjust the dark current knob to zero the needle Rotate sensitivity knob fully clockwise Set slits to 2 mm Turn shutter switch ON Rotate wave length knob toward shorter wave length (around 300 millimicrons) to zero the needle Turn the upper lamp adjusting screw *slightly* until movement of the needle to the left indicates that more light is entering the monochromator Rebalance the instrument by rotating wave length knob to shorter wave lengths to zero the needle Then again adjust the lamp screw until needle is deflected to the left Continue this procedure of alternate adjustment of screw and wave length knob until further adjustment of the screw does not cause deflection of the needle to the left Next follow this adjusting procedure with the lower lamp adjusting screw then with the condensing mirror screw and finally with the entrance mirror screw Then repeat the entire procedure several times for each of the four screws until the wave length scale cannot be set to any lower value The instrument should then be in optimum focus

m Focusing of Hydrogen Lamp

(1) *Attach the hydrogen lamp housing to the monochromator mounting plate*

(2) Unscrew the four knurled thumbscrews on the end of the phototube compartment and detach the phototube and absorption cell compartments Remove the four machine screws in the corners of the filter slide block and detach it holding the filter slide in one hand to prevent it from being damaged by falling out

(3) Turn on the hydrogen lamp Hold a piece of white paper directly in front of the entrance window (lower) of the monochromator and at right angles to the light beam coming from the condensing mirror in the lamp house Using a small screw driver turn the adjusting screw in the bushing on the left side of the lamp house until the crescent shaped image on the paper falls about [illegible] mm from *the surface of the monochromator mounting plate Using the upper screw* on the back of the lamp house raise or lower the image until it is approximately on a level with the entrance window Focus the beam with the lower screw on the back of the lamp house so that the crescent is narrowest opposite the entrance window as determined by moving the paper back and forth along the light beam If the range of the adjusting screws is insufficient to make the above adjustments it may be necessary to readjust the position of the hydrogen lamp in its holder or to loosen the two mirror clamping screws on the left side of the lamp house and readjust the mirror position. CAUTION DO NOT TOUCH THE MIRROR SURFACE

(4) Replace the filter slide block being careful to assemble the filter slide so that its detent functions properly

(5) Push the filter slide in open the slits to a width of 2 0 mm and set the wave length scale for 450 millimicrons Placing the eye at the hole in the filter slide block look through the exit slit into the interior of the monochromator If the focusing adjustment is approximately correct an image of the quartz prism will be seen in the collimating mirror illuminated with an intense blue light Again adjust the lamp house screws, also the entrance mirror adjustment screw (which

is accessible through the hole in the front of the filter slide block) until the illumination is most intense and almost completely fills the aperture of the prism.

(6) Reassemble the phototube and absorption cell compartments. Place the selector switch in the "Check" position turn the sensitivity knob fully clockwise push in the filter slide and pull out the phototube slide With the shutter "Off" adjust the dark current control in the usual manner and then place the shutter in the "On" position With the slits set at 2.0 mm rotate the wave length scale toward the shorter wave lengths until the galvanometer is brought to balance Readjust the screw on the left side of the lamp house to make the galvanometer needle go off the scale toward the left Rebalance the galvanometer by turning the wave length scale to a shorter wave length Repeat this process so long as the galvanometer needle can be moved to the left by adjusting the screw Then using the other two lamp house adjustment screws continue the process until optimum focus is reached i e when turning any screw in either direction causes the galvanometer needle to go to the right

( ) When the optimum focus is reached by the procedure of Paragraph 6 the wave length scale should read less than 230 millimicrons A high wave length reading indicates (1) one or more of the aluminized mirrors in the light path has deteriorated (2) the radiation output of the hydrogen lamp is below standard or (3) the phototube sensitivity is low The mirrors most subject to deterioration are the concave mirror in the lamp house and the small flat entrance mirror These may be replaced or returned for resurfacing (Inspection under visible light does not necessarily indicate reflecting quality in the ultraviolet)

n. Wave Length Calibration

Because of its numerous intense spectral lines a mercury arc in an ultraviolet transmitting envelope is a useful radiation source for checking the wave length scale of the monochromator

The hydrogen discharge lamp may be used to check the wave length scale at the hydrogen alpha line (656.3 millimicrons) which is emitted with moderate intensity

Using a source of spectral lines the wave length scale may be checked as follows

(1) Place selector switch in "1 0" position and the sensitivity control in the middle of its range Push the filter slide in and select the appropriate phototube Adjust for dark current in the usual manner

( ) Set the transmission scale at about 30% and place shutter switch in the "On" position.

(3) Adjust the wave length scale to give maximum galvanometer deflection to the left If necessary reduce slit width to keep the galvanometer needle on the scale

(4) If the wave length scale reading at maximum deflection differs from the known wave length of the spectral line by more than 0 3 millimicron, adjustment may be made as follows

Set the wave length scale to read the correct wave length of the spectral line Remove the snap button on the left hand end of the monochromator and turn the adjusting screw to give maximum galvanometer deflection. A 5/16" socket wrench is most convenient for turning the screw although a screw driver may be used This adjustment has the effect of changing all readings on the wave length scale by the same angular amount. Consequently in terms of millimicrons it produces a larger effect at the long wave lengths where the scale is compressed. After making the adjustment repeat the wave length check to make certain that the best adjustment has been obtained.

o. Photometric Calibration

The photometric scale is checked by using a series of four filters the transmittances of which have been accurately determined by the National Bureau of Standards.

These four filters are designated as carbon yellow," "cobalt blue' copper gre n and selenium orange The reported transmittances of these filters are in the range from 390 mμ 750 mμ The temperature of the filters should be held as close to 25 C as possible

3 Operating the Instrument

a Securely attach the battery clamps –1 +2 and +6 to the corresponding voltage terminals of the storage battery
b Turn the lamp switch ON "
c Set selector switch to CHECK
d Rotate wave length knob until desired scale value is shown under hairline
e Select proper phototube and filter if required.
f Turn shutter switch OFF
g Rotate dark current knob to zero the meter needle This adjustment must be re peated occasionally (for highest accuracy between each transmission reading) Remove cell compartment cover and put filled cells in place
h Make sure that the cells and holder are seated properly then replace the compart ment cover and place the solvent or blank ' in the light path
i Turn shutter switch ON This switch must be turned gently otherwise the phototube housing may be jarred resulting in needle shift or unsteadiness
j Rotate slit knob to approximately zero the needle or to give the desired slit width
k Rotate the sensitivity knob to zero the needle accurately For highest accuracy this adjustment should be checked between each transmission reading
l Operate the slider knob to place an unknown sample in the light beam
m Set selector switch to 1 0 If the transmission is less than 10% set selector switch to 0 1
n Rotate transmis ion knob to zero the needle
o Record the transmittance or density reading
p Place the next unknown sample in the light path and zero the needle by rotating transmission knob

D FLUOROMETRY—GENERAL PRINCIPLES

References

Radley J A and Grant J Fluorescence Analysis in Ultraviolet Light ed. 3 London 1939 Chapman and Hall Ltd
Dake H C and De Ment J Fluorescent Light and Its Applications New York 1941 Chemical Publishing Company
De Ment J Fluorescent Chemicals and Their Application New York 194? Chem ical Publishing Company

1 Definition

As the term is used in biochemical analysis fluorescence ' means the emission of visible light by a substance when it is irradiated with ultraviolet light If emission of visible light occurs after the removal of the ultraviolet light the afterglow is termed ' phosphorescence '

2 Laws of Fluorescence

Fluorometry is based on the general laws of photochemistry and there are three specific 'laws of fluorescence '

a. Energy must be absorbed by a luminescent system before emission can occur (the absorption law of De Ment)

b In a fluorescent system the emitted wave lengths are longer than the absorbed wave lengths that is the energy emitted is less than the energy absorbed (the emission law of Stokes)

c The absorption of radiation by a luminescent system is a quantum process involving one quantum per ab orbing unit (e g atom or center) the yield being unity only in the ideal case (one aspect of the photochemical equivalence law of Einstein)

3 General Principles of Fluorometry

All quantitative fluorometry is based on a fundamentally simple system A source of ultraviolet light casts a beam on the solution to be examined Visible light is emitted and is measured either visually (against a comparison standard) or photoelectrically

In general it is necessary to conduct fluorometry with dilute solutions which are measured against calibration curves prepared under conditions identical with those of the analysis The reason for this is that the absolute intensity of fluorescence is highly variable depending upon many important factors including temperature time of exposure nature of instrument traces of impurities pH and quenching Quenching ' is a complex phenomenon of which there are at least three kinds (1) interference of a molecule's fluorescence by other organic molecules (2) interference by inorganic molecules (3) and reabsorption of visible light by other molecules of the same compound as those being measured Fluorometry by its nature is not as capable of extreme accuracy and reproducibility as is spectrophotometry Every kind of analysis is strongly influenced by the nature purity history and condition of the material under analysis

E PHOTOFLUOROMETRY—THE COLEMAN PHOTOFLUOROMETER MODEL 12B

1 General Construction of the Photofluorometer

This instrument consists basically of (a) a mercury vapor lamp source of ultraviolet light (b) a series of filters to transmit a beam of ultraviolet light of the appropriate wave length (c) a cuvette to hold the solution to be analyzed in the beam of ultraviolet light (d) at right angles to the beam of ultraviolet light where it passes through the solution a filter to transmit the beam of visible light in the appropriate range of wave length (e) a photo cell in the path of the visible light (f) an amplifying system and a direct reading galvanometer to measure the potential from the photocell (See Fig 7 for general construction)

2 Setting Up and Adjusting the Instrument

a. Location

Place the instrument in any convenient location and plug into 110 1 0 volt 60 cycle supply line first insuring that the ' STD knob is in the extreme counterclockwise position Do not attempt to operate the instrument except at the frequency and voltage specified. It is wise to place rubber pads under the *four feet* to minimize vibration conducted through the desk

These four filters are designated as carbon yellow, cobalt blue copper green and selenium orange ' The reported transmittances of these filters are in the range from 390 mμ 750 mμ The temperature of the filters should be held as close to 25 C as possible

3 Operating the Instrument

a Securely attach the battery clamps –1 +2 and +6 to the corresponding voltage terminals of the storage battery
b Turn the lamp switch ON
c Set selector switch to CHECK
d Rotate wave length knob until desired scale value is shown under hairline
e Select proper phototube and filter if required
f Turn shutter switch OFF
g Rotate dark current knob to zero the meter needle This adjustment must be repeated occasionally (for highest accuracy between each transmission reading) Remove cell compartment cover and put filled cells in place
h Make sure that the cells and holder are seated properly then replace the compartment cover and place the solvent or blank in the light path
i Turn shutter switch *ON* This switch must be turned gently *otherwise the* phototube housing may be jarred resulting in needle shift or unsteadiness
j *Rotate slit knob to approximately zero the needle or to give the desired slit* width
k. Rotate the sensitivity knob to zero the needle accurately For highest accuracy this adjustment should be checked between each transmission reading
l Operate the slider knob to place an unknown sample in the light beam
m Set selector switch to 1 0 If the transmission is less than 10% set selector switch to 0 1
n Rotate transmission knob to zero the needle
o Record the transmittance or density reading
p Place the next unknown sample in the light path and zero the needle by rotating transmission knob

D FLUOROMETRY—GENERAL PRINCIPLES

References

Radley J A and Grant J Fluorescence Analysis in Ultraviolet Light ed. 3 London 1939 Chapman and Hall Ltd
Dake H C and De Ment J Fluorescent Light and Its Applications New York *1941* Chemical Publishing Company
De Ment J Fluorescent Chemicals and Their Application New York 194 Chemical Publishing Company

1 Definition

As the term is used in biochemical analysis fluorescence" means the emission of visible light by a substance when it is irradiated with ultraviolet light If emission of visible light occurs after the removal of the ultraviolet light the afterglow is termed phosphorescence '

2 Laws of Fluorescence

Fluorometry is based on the general laws of photochemistry and there are three specific ' laws of fluorescence '

a. Energy must be absorbed by a luminescent system before emission can occur (the absorption law of De Ment)

b In a fluorescent system the emitted wave lengths are longer than the absorbed wave lengths that is the energy emitted is less than the energy absorbed (the emission law of Stokes)

c The absorption of radiation by a luminescent system is a quantum process involving one quantum per absorbing unit (e g atom or center) the yield being unity only in the ideal case (one aspect of the photochemical equivalence law of Einstein)

3 General Principles of Fluorometry

All quantitative fluorometry is based on a fundamentally simple system A source of ultraviolet light casts a beam on the solution to be examined Visible light is emitted and is measured either visually (against a comparison standard) or photoelectrically

In general, it is necessary to conduct fluorometry with dilute solutions which are measured against calibration curves prepared under conditions identical with those of the analysis The reason for this is that the absolute intensity of fluorescence is highly variable depending upon many important factors including temperature time of exposure nature of instrument traces of impurities pH and quenching ' Quenching i a complex phenomenon of which there are at least three kinds (1) interference of a molecule s fluore cence by other organic molecules (2) interference by inorganic mole cules (3) and reabsorption of visible light by other molecules of the same compound as those being measured Fluorometry by its nature is not as capable of extreme accuracy and reproducibility as is spectrophotometry Every kind of analysis is strongly influenced by the nature purity history and condition of the material under analysis

E PHOTOFLUOROMETRY—THE COLEMAN PHOTOFLUOROMETER MODEL 12B

1 General Construction of the Photofluorometer

This instrument consists basically of (a) a mercury vapor lamp source of ultraviolet light (b) a series of filters to transmit a beam of ultraviolet light of the appropriate wave length (c) a cuvette to hold the solution to be analyzed in the beam of ultraviolet light (d) at right angles to the beam of ultraviolet light where it passes through the solution a filter to transmit the beam of visible light in the appropriate range of wave length (e) a photo cell in the path of the visible light (f) an amplifying system and a direct reading galvanometer to measure the potential from the photocell (See Fig 7 for general construction)

2 Setting Up and Adjusting the Instrument

a. Location

Place the instrument in any convenient location and plug into 110 120 volt 60 cycle supply line first insuring that the STD knob is in the extreme counterclockwise position Do not attempt to operate the instrument except at the frequency and voltage specified. It is wise to place rubber pads under the four feet to minimize vibration conducted through the desk

b *Preliminary Adjustments*

Before mak ng tests with th s nstrument it s essent al that both ts mercury vapor lamp and electron c ampl fier be turned on and allowed to reach equ l brium Proceed as follows

(1) Plug extens on cord nto a 110 1?0 volt AC supply l ne

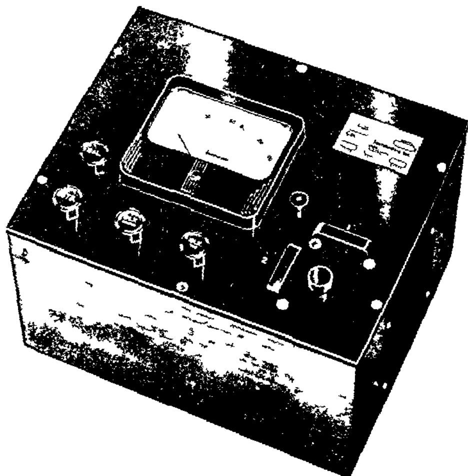


Fig 7—The Coleman Photofluorometer Model 1 B (Reprodu ed by permission of the manufacturers

(2) Insert n oblong panel open ng #1 the proper filter for the measurement nvolved e g use filter *B* for th amine or use filter B_2 when cons dering r boflav n *These* two part cular filters pass the 365 m ll m cron and 4 6 m ll m cron me cury bands respect vely The filtered beam s then projected to focus with n the ¾ round cuvette Filter B is also fitted with a ?9% transm ss on screen to reduce the l ght ntensity to a level at wh ch s gn ficant bleach ng of th ochrome does not occur

(3) In oblong panel opening #? insert the correspond ng filter us ng PC-1 when measur ng th amine and filter PC ? when exam n ng riboflavin The purpose of

these second filters is to screen the phototube from the waveband passed by the corresponding first filter while allowing th fluorescent light of the sample to enter the phototube and be measured.

(4) Energize the instrument by rotating the STD knob clockwise until it just cl cks This turns on the amplifier and also starts the cooling fan and excites the mercury vapor lamp

(5) After 0 seconds continue rotation of the STD knob to extreme clockwise position This adjusts the amplifier to maximum sensitivity (The STD knob thus serves both to turn the instrument on and off as well as to adjust its sensi tivity)

(6) Depre s button B and while it is in the depres ed position adjust knob BAL until the in trument meter reads approximately zero The purpose of this ad ju tment is to cond tion the electronic ampl fier to the condition of the A bat teries The adjustment is not at all critical but should be checked occasionally durir g prolonged tests a ways again checking BLK as in (f)

(7) Adjust the BLK knob until the instrument meter reads zero This knob serves initially to balan e the amplifier to zero as indicated above and may sub s quently be used automatically to correct for fluorescence of blanks during actual analysis of fluorescent samples

(8) Now allow at least ten minutes for the instrument to stabilize and then repeat steps (6) and (7) (Note that the mercury vapor lamp once turned off has to cool down before it will light again)

3 Making Routine Measurements

For every run of unknowns certain steps are necessary These are (a) setting sensitivity to a fixed value against a stable reference standard (b) reading a reagent blank and (c) reading the unknowns The following example is for thiamine by the thiochrome method Other substances may require other stable reference standards with an emission similar to that of the unknown

a. Complete adjustments (1) through (8) in section 2 b above

b Be sure that enough standard cuvettes are available to carry out all read ngs ex peditiously (This fluorometer accepts ordinary $^{3}_{4}$ round test tubes and is so designed optically that the tube d ameter is not critical Use not less than 8 ml of sample and be sure that tubes are covered to exclude light during all tests)

c Pla e cuvette of stable reference solut on in cuvette well cover with cap and then open the lamp shutter by fully depress ng button P The beam of filtered UV light projected into the cuvette excites fluorescence and the resulting light enter ing the phototube causes the instrument meter to re i ter With P fully de pressed adjust the STD knob until the meter reads a value consist nt with the concentration of the sample to be exami ed This operation constitutes the sensitivity adjustment Note the value of the reference standard and immediately before testing each sample first insert the reference solution and then adj ist the meter to read the value of the reference standard when button P is de pressed. This stable reference solution is nece sary because the instrument s sens tivity is dependent in part on the inten ity of the mercury vapor lamp wh ch in turn depends on the voltag and frequency of the power supply Since the e latter factors may vary with time it is advisable to check the sensitivity o the photo-fluorometer immediately before each test by insert ng in the cuvette well a stable referen e solution e g qu nine sulfate when exam ning thiamine A solution con tain ng 0 $^{\circ}$5 mg of quin ne sulfate dissolved in one liter of N/10 sulfuric acid is set at 60 on the scal It is re ommended that this stabl reference solution be utilized solely as a means of checking and maintaining the instrument sensitivity

at the selected value The practice of assigning a definite value to the ratio of instrument responses to quinine and to vitamin is unnecessary and of questionable merit A much more reliable calibration consists of running a calibration curve with standard vitamin solutions

d Insert a cuvette *containing a reagent blank cap the cuvette fully depress button* P and read the meter as soon as the pointer has come to rest (The glassware solvent and the other chemicals used for preparing the sample may themselves ex hibit some measure of fluorescent response and the activity of these blank' constituents must therefore be considered in all measurements In practice the blank solution is prepared by combining in the same manner and amounts all of the ingredients of the corresponding ample *solution except the considered constituent* In the determination of thiamine the fluorescent compound is thiochrome formed by oxidizing thiamine with potassium ferricyanide In this instance the blank'' solution is prepared exactly the same as the sample using water in place of the sample to be analyzed. Thus the blank is slightly fluorescent The corresponding meter deflection caused by the thiochrome is equal to the difference between the meter deflection of the blank and the deflection occasioned by the corresponding sample)

e Set the sensitivity of the instrument with the stable reference standard as in step (c) above

f Now read an unknown Insert a cuvette containing a solution of the considered constituent (e g thiochrome for thiamine) of approximately the concentration to be considered in subsequent te ts Cap the tube fully depress button P ' and then read the meter as soon as its pointer comes to rest (The best accuracy is obtained when the range of concentration to be examined gives a convenient deflec tion and it is good practice not to attempt measurements at very high concentra tions since the fluorescence concentration curves depart from linearity at high con centrations For example the recommended range of concentration for thiamine is not more than 2 gamma oxidized to thiochrome in 10 ml of isobutanol Higher concentrations should be diluted to this range)

g Before all subsequent readings set the sensitivity of the instrument by means of the stable reference standard.

4 Computation of Results

a. Calibration Curves

We recommend the use of a calibration curve derived from samples of standards run through the analytical procedure (The reason for this recommendation is that fluorescence may be affected markedly by slight alterations in analytical conditions Hence computation of a standard K i e units of solution concentration per meter scale unit is inherently liable to error In addition the fluorescence galvanometer *reading* relation is not always linear)

b Calculated Constant

We do not recommend this calculation for the reasons stated above However it is given here for the sake of completeness

(1) Let K = units of solution concentration per scale division Let A and B be scale readings for two known concentrations of the substance under consideration S_1 and S respectively Then $K = \frac{B - A}{S_1 - S}$

(2) Let K = constant as defined above
U = scale reading for unknown
B = scale reading for blank

Then
Concentration of unknown = K × (U − B)

5 Adjustment and Replacement of Parts

a. Adjustment of Mercury Vapor Lamps Autotransformer to Match Exciting Voltage

The H 4 mercury vapor used in the Coleman Model 1_ photofluorometer is adjusted to operate at 1?0 volts when shipped If with this adjustment it is connected to a higher supply voltage the intensity of the lamp will be very markedly increased and there will be an appreciable reduction of the lamp life In instances when it is nece sary to use this lamp in conjunction with a line or voltage regulator delivering other than 1 0 volts the connections on the autotransformer must be changed to fit the alternate voltage This adaption can be made by the following procedu e

(1) Remove the two screws which secure the cover on the small autotransformer

(2) On the terminal board inside this transformer will be found terminals marked 110 115 and 120 The instrument is shipped with a green lead con nected to the 120 terminal

(3) If the supply voltage is between 108 and 113 volts move this lead to the 110 terminal (This same connection will also assure maximum possible brilliance when it is necessary to operate at less than 108 volts) If the supply voltage is between 113 118 volts connect the green lead to the terminal marked 11_ If the supply voltage is above 118 volts leave the green lead connected to the terminal marked 1 0

(4) After making the proper connection be sure that all terminals are s rewed tight and replace the cover The instrum nt is now ready for ope ation

(5) Transformers are individually adjusted to insure the maximum possible voltage control and as a result the output voltage of a given transformer may ex eed 115 volts Therefore when us ng the transformer be sure to ascertain its actual out put voltage which is g ven on a plate attach d to the front of the unit and then ad just the lamp autotransformer to correspond

b Replacement of Mercury Vapor Lamp

The lamps used in the Model 1 is an AH 4 me lium high pressure quartz mercury arc The life expectancy of this lamp is in the neighborhood of 1000 hours When failure occurs as evidenced by unsteadiness of illumination obscuring of the walls of the central quartz capillary and finally refusal to start when lamp has cooled re placement should be made with another lamp of exactly the same type Specify serial number of instrument when ordering

(1) Remove the five screws around the edge of the panel

(?) Place the instrument at the edge of the table with the left ha d side of the box far enough off the table so that the hole in the bottom from which the rubber covered cable to th transformer issues is just clear of the edge of the table This will free the cable and fac litate removal of the panel.

(3) Pull the panel straight upward until ch cke l by the battery cable Bring the back edge of the panel up and forward and lay it on its face on the top of the box which should be move l back onto the table

(4) The mercury lamp is secured by two spring retaining straps which hold it to an aperture plate Connect ons are made by twisted leads which connect to a ter minal str p at the s de of the chassis adjacent to the lamp

(5) Disconnect the leads from the terminal strip

(6) Loosen the nuts which hold down the lamp retaining straps It is not gen erally necessary to remove the straps With the straps loose slide the lamp end wise till clear of the straps.

(7) Insert a new lamp into the straps with the small conical tip on the inner quartz capillary pointed away from the aperture plate and the two support rols inside the lamp approximately equi listant from the plate Center the lamp by slid ng

endwise so that only the transparent part of the capillary is in line with the three-quarter inch hole in the aperture plate the metalized end caps being clear of the hole

(8) Push down the retaining straps and pull the nuts down gently not tightly A gentle pressure here will hold the lamp firmly, excessive pressure may shatter the outer tube of the lamp

(9) Slide the spade lugs on the connecting leads under the terminal screws and tighten Be sure that spade lugs do not touch each other or touch the rivets holding the terminal strip to the chassis

(10) Replace the panel by repeating instructions 1, 2 and 3 in reverse (Note Be sure battery cable does not crowd over so as to interfere with fan or mercury lamp)

c Replacement of Vacuum Tube

The vacuum tube used in the Model 12 has been carefully selected and rigorously tested before incorporation in the instrument Although a standard radio tube of the correct type may be used in this instrument it is advisable to secure replacement tubes through a Coleman dealer When ordering a replacement tube always specify the serial number of the instrument Symptoms of tube failure are (1) inability to reach the proper adjustments even though the batteries check well, and (2) when filament burns out continuous off scale reading to the right not responsive to position of the BAL or BLK knobs When it becomes necessary to replace the vacuum tube

(1) Remove panel as described in previous sections

(2) The va uum tube is surrounded by a perforated metal shield with a removable end cap Remove this cap exposing the grid connection to the tube Pull the grid connection off the tube Remove the perforated shield by pulling it up off the tube The tube may now be removed from its socket

(3) After inserting the new tube be sure to remove any conducting film existing on outside of bulb between grid terminal and base by wiping gently with tissue or a clean cloth before replacing the shield Replace the grid connection before placing the end cap on the shield being careful to avoid again touching glass

(4) Replace panel as described in previous section

d Replacement of Batteries

The Model 12B requires the following batteries for its operation

One 45 v B Battery type 5308
One 3 v A Battery type 2F2H
One 90 v C Battery type Z60BP
One 6 V Bucker Battery type F4X

The life expectancy of the 2F2H should be above 400 hours of service that of the F4X about 1000 hours The other two batteries should be good for over a year Failure of the 2F2H or the #5308 will be shown by inability of the BAL knob to bring the meter up to zero while failure of the F4X will be shown by the inability of the BAL knob to bring the meter down to zero The Z60BP should be replaced when its voltage drops below 50 volts When it becomes necessary to change batteries

(1) Remove the five screws around the edge of the panel and disassemble as described in previous sections

(2) Dis onnect battery to be replaced. It is wise also to check the voltage of all the batteries and replace any which show a voltage less than three fourths of their rated voltage

(3) Remove screws securing holddown strap of battery to be replaced. Remove strap thus freeing battery (The Z60BP is held down by a wooden block instead of a strap)

(4) Replace battery with identical battery Replace strap and reconnect battery To facilitate this the following table gives the color coding of the battery cable

COLOR		CONNECTION
Gray	−A	(F-H)
Black	+A	(F H)
Blue	−B	(308)
Yellow	+⅞ V B	(5308)
Red	+45 V B	(5308)
Brown	−C	(Z60BP)
Green	+C	(Z60BP)

The leads to the F4X battery are separate comprising a twisted red and black pair the red being plus

(5) Replace panel as described in previous section

F FLUOROMETRIC METHODS FOR QUALITATIVE USE AND FOR QUANTITATIVE MEASUREMENTS IN THE FIELD—VISUAL FLUOROMETRY

Principle

Qualitative fluorometry is best done by eye because it is necessary to distinguish fine shades of color No photoelectric fluorometer of which we are aware is suitable for qualitative use

For many quantitative purposes in the field visual fluorometry is equally as satisfactory as photoelectric fluorometry The chief reason is that under field conditions line voltage is often extremely erratic This impairs the accuracy of photoelectric fluorometers but has no effect on the accuracy of visual fluorometry

Apparatus

1 A suitable darkened room for reading unknown solutions This need not be completely light tight We have used successfully x ray rooms photographic dark rooms bath rooms abandoned kitchen cupboards and U S Army command post tents

2 A source of ultraviolet light consisting of a General Electric Type H 4 mercury vapor bulb enclosed in a box with louvers for air cooling and a Wood filter for absorbing visible light A General Electric Autotransformer Type 59G18 is installed between the bulb and any suitable source of 110 volt AC In the field we have used simple motor generators without line stabilization

3 The fluorometric comparator consisting of a thin wooden rack containing 10 Corning glass stoppered thin walled ~ ml test tubes outside diameter 10 mm and length 75 mm Holes are bored so that these tubes which contain standard solutions are flanked by empty holes to contain 10 × 75 mm thin walled test tubes containing the unknown solution The rack is constructed so as to slide conveniently in a direction perpendicular to the ultraviolet ray and 8 cm from the Wood filter

4 Suitable beakers delivery pipettes and other usual appurtenances including a large number of 10 × 75 mm thin walled test tubes One special pipette is of great assistance This is an automatic syringe pipette the use of which is described in the section on volumetric apparatus

5 A small spotlight in the darkened room in such a position that a recorder can easily take down notes without disturbing the operator who is doing the fluorometry

Reagents

1 These are different for different estimations but in all cases thoroughgoing reagent blanks must be run on each new batch and if there is a perceptible blank the reagents must be either replaced or purified until the blank is negligible

2 Standard solutions are prepared by running solutions of known concentration through the whole method The final solutions are placed in the glass stoppered 2 ml test tubes up to a convenient level usually 1 ml, and the wooden racks are clearly labeled in fluorescent paint or ink with a number equivalent to the concentration of unknown substance in 100 ml of original solution This procedure automatically takes care of calculations and also includes any correction necessary for systematic losses in the methods The preparation of standard solutions will be described under individual methods

Procedure

1 Each method is carried out as described in detail in subsequent sections

2 As the final step in each method the compound to be measured is extracted into an appropriate solvent and the solvent layer is separated from the aqueous layer, either by simple standing or by centrifuging

3 An automatic syringe pipette fitted with a long stainless steel needle is stopped at approximately 1 ml. The tubes containing samples in which the solvent layer has been separated from the aqueous layer are placed in convenient racks in front of the operator Other racks containing clean 75 × 10 mm test tubes in positions corresponding to the unknowns are similarly placed

4 Approximately 1 ml of solvent is transferred to a 75 × 10 mm test tube Excess solvent in the automatic syringe pipette is removed by several up and down motions of the plunger and the next sample is transferred The transfer pipette need be rinsed with blank solvent only when it is expected that there will be very wide variations in concentration from unknown to unknown e g if fasting and loaded samples are being run at the same time

5 The rack containing 75 × 10 mm test tubes is now transferred to the darkroom after the notebook is appropriately ruled and after the ultraviolet bulb has been turned on for at least 10 minutes to allow thorough warming up

6 After entering the darkroom allow about 5 minutes for the eyes to become adapted to the dark

7 Comparison is made by inserting the unknown solution from empty hole in the comparator until a match is obtained. It is usually wise to commence reading with the unknown in position between two standard solutions both of which are unquestionably brighter than the unknown and then to move the unknown down the rack for final matching

8 The recorder should record the value for the unknown in the appropriate place in the notebook It will be recalled that these readings represent without further calculation the concentration of the original unknown solution

Calculations and Examples

1 These will be discussed individually in individual methods

Precautions

1 Some of the standard solutions are themselves unstable and should be checked daily or even oftener against freshly prepared standards This is particularly true of riboflavin Quinine is apparently stable indefinitely under usual conditions but we have seen quinine standards deteriorate in very hot weather

2 The standard solutions and unknown solutions must be at approximately the same temperature during reading because fluorescence is markedly affected by changing the temperature of the solution The temperature will be the same if reagents samples and standards are kept on the same bench

G FLAME PHOTOMETRY—THE BECKMAN FLAME PHOTOMETER AT TACHMENT

1 General Principles of Flame Photometry

The purpose of flame photometers now commercially available is to intro duce inorganic substances into a flame at a uniform rate so that light pro duced thereby will be constant The flame used is very hot so that the spec tral lines are excited much more strongly than with a cool flame and more lines of more elements can be excited to a usable intensity than with an or dinary cool flame In this way it is possible to make instantaneous spectro photometric measurements whereas the usual method requires a spectrogram followed by a densitometer measurement and the results have to be inter preted with the aid of sensitometry measurements made on each plate at all wave lengths used *A flame source has certain advantages over the customary* arc and spark sources for chemical analysis Unnecessary lines are not ex cited the temperature is lower so the continuous background is lower the temperature and rate of feed of specimen can be held constant over a long period allowing numerous individual line measurements to be made in succes sion the flame and rate of feed can be exactly reproduced at any time being extended in area the flame does not require precise optical alignment and slight sidewise motions do not alter the luminosity and especially the speci men to be analyzed is introduced into the region of excitation at a uniform rate with the more volatile and less volatile constituents in perfect admixture (In other sources the more volatile constituents vaporize first so that photo graphic or integrating methods must be used and the sample must all be con sumed for accurate determinations)

Of the several good instruments now on the market we shall describe in detail only one the flame photometer attachment of the Beckman spectropho tometer

2 General Construction of the Beckman Flame Photometer

a. Photometer Attachment

With the flame photometer attachment the Beckman Type DU Spectrophotometer becomes an emiss on spectrophotometer and pe cent transmittance becomes light in tensity Basically there are four separate parts (see Fig 8)

(1) The Beckman Type DU Spectrophotometer for measuring the intensity of emitted l ght Some modificat ons are required as described below

( ) A burner to produce emission from the sample The burner of this instrument burns illuminating gas with air or oxygen the mixture emerging from 30 ports Through the burner seven ducts carry fine droplets of m st containing the speci men under analysis The burner is of stainless steel and is water cooled. A water cooled chimney which may be extended out of doors surrounds the flame and pro tects against heat and fre hazard. The burner and chimney are arranged as an integral unit with gas oxygen and water cool ng l nes going to the control unit and two vapor ducts going to the two sprayers One of the e ducts is for unknown specimens the other for standard solutions.

Reagents

1 These are different for different estimations but in all cases thoroughgoing reagent blanks must be run on each new batch and if there is a perceptible blank the reagents must be either replaced or purified until the blank is negligible

2 Standard solutions are prepared by running solutions of known concentration through the whole method The final solutions are placed in the glass stoppered 2 ml test tubes up to a convenient level usually 1 ml and the wooden racks are clearly labeled in fluorescent paint or ink with a number equivalent to the concentration of unknown substance in 100 ml of original solution This procedure automatically takes care of calculations and also includes any correction necessary for systematic losses in the methods The preparation of standard solutions will be described under individual methods

Procedure

1 Each method is carried out as described in detail in subsequent sections

2 As the final step in each method the compound to be measured is extracted into an appropriate solvent and the solvent layer is separated from the aqueous layer either by simple standing or by centrifuging

3 An automatic syringe pipette fitted with a long stainless steel needle is stopped at approximately 1 ml The tubes containing samples in which the solvent layer has been separated from the aqueous layer are placed in convenient racks in front of the operator Other racks containing clean 75 × 10 mm test tubes in positions corresponding to the unknowns are similarly placed

4 Approximately 1 ml of solvent is transferred to a 75 × 10 mm test tube Excess solvent in the automatic syringe pipette is removed by several up and down motions of the plunger and the next sample is transferred. The transfer pipette need be rinsed with blank solvent only when it is expected that there will be very wide variations in concentration from unknown to unknown, e g if fasting and loaded samples are being run at the same time

5 The rack containing 75 × 10 mm test tubes is now transferred to the darkroom after the notebook is appropriately ruled and after the ultraviolet bulb has been turned on for at least 10 minutes to allow thorough warming up

6 After entering the darkroom allow about 5 minutes for the eyes to become adapted to the dark

7 Comparison is made by inserting the unknown solution from empty hole in the comparator until a match is obtained It is usually wi e to commence reading with the unknown in position between two standard solutions both of which are unquestionably brighter than the unknown and then to move the unknown down the rack for final matching

8 The recorder should record the value for the unknown in the appropriate place in the notebook It will be recalled that these readings represent without further calculation the concentration of the original unknown solution

Calculations and Examples

1 These will be discussed individually in individual methods

Precautions

1 Some of the standard solutions are themselves unstable and should be checked daily or even oftener against freshly prepared standards This is particularly true of riboflavin Quinine is apparently stable indefinitely under usual conditions but we have seen quinine standards deteriorate in very hot weather

2 The standard solutions and unknown solutions must be at approximately the same temperature during reading because fluorescence is markedly affected by changing the temperature of the solution The temperature will be the same if reagents samples and standards are kept on the same bench

G FLAME PHOTOMETRY—THE BECKMAN FLAME PHOTOMETER ATTACHMENT

1 General Principles of Flame Photometry

The purpose of flame photometers now commercially available is to introduce inorganic substances into a flame at a uniform rate so that light produced thereby will be constant The flame used is very hot so that the spectral lines are excited much more strongly than with a cool flame and more lines of more elements can be excited to a usable intensity than with an ordinary cool flame In this way it is possible to make instantaneous spectrophotometric measurements whereas the usual method requires a spectrogram followed by a densitometer measurement and the results have to be interpreted with the aid of sensitometry measurements made on each plate at all wave lengths used A flame source has certain advantages over the customary arc and spark sources for chemical analysis Unnecessary lines are not excited the temperature is lower so the continuous background is lower the temperature and rate of feed of specimen can be held constant over a long period allowing numerous individual line measurements to be made in succession the flame and rate of feed can be exactly reproduced at any time being extended in area the flame does not require precise optical alignment and slight sidewise motions do not alter the luminosity and especially the specimen to be analyzed is introduced into the region of excitation at a uniform rate with the more volatile and less volatile constituents in perfect admixture (In other sources the more volatile constituents vaporize first so that photographic or integrating methods must be used and the sample must all be consumed for accurate determinations)

Of the several good instruments now on the market we shall describe in detail only one the flame photometer attachment of the Beckman spectrophotometer

2 General Construction of the Beckman Flame Photometer

a Photometer Attachment

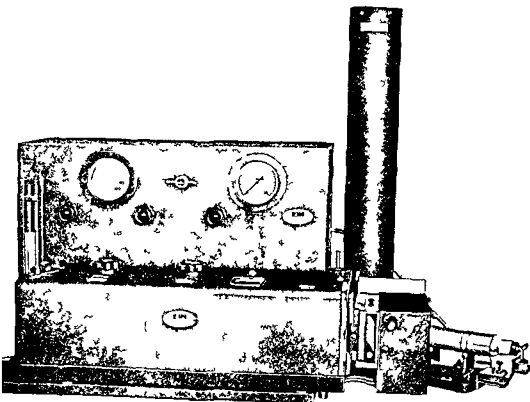
With the flame photometer attachment the Beckman Type DU Spectrophotometer becomes an emission spectrophotometer and percent transmittance becomes light intensity Basically there are four separate parts (see Fig 8)

(1) The Beckman Type DU Spectrophotometer for measuring the intensity of emitted light Some modifications are required as described below

(2) A burner to produce emission from the sample The burner of this instrument burns illuminating gas with air or oxygen the mixture emerging from 30 ports Through the burner seven ducts carry fine droplets of mist containing the specimen under analysis. The burner is of stainless steel and is water cooled. A water cooled chimney which may be extended out of doors surrounds the flame and protects against heat and fire hazard The burner and chimney are arranged as an integral unit with gas oxygen and water cooling lines going to the control unit and two vapor ducts going to the two sprayers One of these ducts is for unknown specimens the other for standard solutions

(3) Sprayers are made of Pyrex glass and are conveniently located to facil tate filling and washing and observat on of the spec mens

(4) The control unit has the following on its panel a gas manometer to read the gas pressure at the burner nozzle an oxygen gauge to read the oxygen pressure at the burner nozzle an a r pressure gauge to read the pressure at the sprayer noz zles *The a r valve is a cut off valve only* it is used e ther open or closed The a r pressure regulator controls the air pressure reduc ng it from 30 to 50 lbs/sq in at input to 15 at output The gas valve controls and cuts off the gas The oxy gen valve controls and cuts off the oxygen



F g 8—The flame photometer attachment for the Beckman Type DU Spectrophotometer (Reproduced w th the permis ion of the manufacturers)

3 Setting Up the Instrument

a. Alterations in the Beckman DU Spectrophotometer

The convers on of the spectrophotometer into an em ss on measuring instrument by add tion of the flame attachment nvolves four small changes n the spectrophotometer

(1) The flame used in em ss on is not as steady in intens ty as the hydrogen or *tungsten lamp used n absorption measurements* The galvanometer fluctuat ons therefore increase cons derably and must be m n mized or smoothed out This is done by changing the ²000 megohm res stor in the phototube compartment to a 10 000 megohm res stor The change of res stors gives the galvanometer five times the prev ous t me lag enabl ng it to be read with a high degree of reproduc bility

(2) An aluminum shield is placed over the phototube compartment to protect it from the heat of the flame

(3) The spectrophotometer has to be raised onto the platform of the control panel.

(4) When the flame attachment is working the entrance mirror of the spectrophotometer has to be adjusted to an angle optimal for measuring the emitted light

b Preliminary Adjustments and General Precautions

(1) Place the combined unit of control panel flame tower and sprayers on a rigid table convenient to sources of gas air water and electricity To avoid erratic results the room in which the instrument is to work must be as free of dust and drafts as possible

( ) Place the spectrophotometer on the base of the control panel and its left end flush with the right margin of the gas manometer

(3) The hoses are attached securely Scotch tape or small clamps serve well The air line must be securely clamped both on the input side and the exits to sprayers The a r pressure must be turned off (gauge reading zero) before removing a hose from the sprayer Otherwise a large air flow will occur and water from the high pressure bubbling towers may be carried through. A drip column has been added to catch a reasonable amount of such water However it can only be drained by removing the back of the cabinet

(4) The water is turned on at about one liter per minute Too high a flow is objectionable Water to drain should be hot enough but not boiling with burner running If too much is run through the bubbling towers may become over filled.

(5) Turn on gas low just enough to light and light it When it is lit turn gas up to the fiducial marks on the manometer Always remember that water must be tu ned on before the flame is l t and the flame must be turned out before the water is turned off

(6) Turn oxygen on to about ᵒ₀ inches of water and check gas pressure on the manometer In case the gas pressure rises too high the flame may back fire as all oxygen flames do This is harmless in the present burner the small mixing chamber in the base of the burner has low pneumatic resistance to the outside air and no damage will result

4 Procedure for Routine Analyses

This laboratory prefers to use direct comparison between unknown samples and aqueous standard solutions rather than an internal standard of lithium added to each unknown specimen Some of the reasons for this choice are to be found in the discussion section below To make a run of analyses

a. Prepare a group of samples the approximate concentrations of which are known properly d luted to bring them within the linear C T range Standards are prepared from a stock standard so that they also be in the correct range The special conditions of slit opening flame temperature etc as determined by trial and error are produced in the instrument and the samples and standards are put into the 5 ml beakers prov ded

b Turn switch to check

c Turn on water

d Plug in atomizer heate

e Light burner low at fi st then high

f Turn on oxygen

g Allow 5 minutes for warming up

h Turn on atomizer, read an unknown sample on the "%T" scale and immediately after two standards whose concentrations i e, "%T" readings lie on either side of the sample Repeat a number of times until all readings are steady and reproducible and good checks are obtained.

i Repeat this process with the rest of the unknown samples

5 Computations

If the graph of "C %T" is linear in the range used then for each sample the concentration can be calculated from the simple ratio (standard galvanometer reading)/(sample galvanometer reading) In this case the ratios of sample reading with either of the standards bracketing it and the reading from the "C %T" standard curve should check. If a determination is done in which it is not possible to obtain linear readings the simplest method of computation is to use a calibration curve prepared from standards When graphing is resorted to, a semilogarithm or logarithmic plot can be used, the criterion being which gives the greater degree of linearity

6 Discussion of Sources of Error in Flame Photometry

a Metals Which Can Be Measured

In clinical chemistry about all the flame photometer is really useful for is estimating sodium, potassium and calcium as shown in Table 1 Other alkali metals and alkaline earths can be measured readily, but for other metals such as copper and iron chemical methods are far more reliable than the flame photometer Since sodium, potassium and calcium are by far the most common metals measured with the flame photometer the rest of this discussion is restricted to them

b Sources of Instrumental Error

Readings for any given sample in the flame photometer may be affected by many variables among which are quality of gas rate of water flow through chimney and burner sensitivity setting of the instrument width of slit wave length setting and pressures of gas oxygen, and air The adjustment of these various components depends not only upon the element to be analyzed but also upon other interfering substances which may be present Therefore no hard and fast rules can be formulated for the operation of the instrument but a few general precautions can be stated to help in obtaining consistent results

(1) In our experience ordinary city gas is too variable in pressure and in quality to be completely satisfactory We have found it necessary to purchase tanks of gas of constant source and composition By means of a suitable reducing valve this gas is led into the instrument With some kinds of such gas it may be necessary to adjust the burner

(2) Water flow should be as constant as possible This can be accomplished by using a reservoir with an overflow thus keeping a constant pressure of water at all times

(3) Even with all variables held constant the readings of a given sample will rise continuously for about ½ hour However, if the standards and samples are read immediately one after the other the determination can be made with good accuracy without waiting for the readings to become exactly constant

(4) The lines radiated by each element vary in intensity those of the alkali metals in general being most intense As intensity increases the slit width can be decreased to obviate interference by other elements When this is done, however

TABLE 1

SENSITIVITY OF FLAME PHOTOMETER WITH DIFFERENT METALS IN HOT FLAME (GAS OXYGEN)

ELEMENT AND COMPOUND	SLIT WIDTH MILLIMETERS	LINES USED ÅNGSTROM UNITS	CONCENTRATION MEASURABLE PARTS/MILLION	DETECTABLE PARTS/MILLION
Sodium NaCl	0 1 0 2	5890 5896	0 5	0 1
Potassium KCl	0 1 0 2	7665	0 5	0 1
Lithium Li SO	0 1 0	6708	0 5	0 1
Cesium Cs_2CrO	0 1 0 ?	85 1	0 5	0 1
Calcium CaCl	0 1 0 3	618 6 03	3	1
Manganese MnCl	0 1 0	4031 4034	2 3	1
Copper CuSO	0 1 0 2	3 48 3274	30	10
Magnesium MgCl	0 1 0 ?	285? 3838	100	25
Cobalt $CoCl_2$	0 1 0 ?	3530 350?	100	25
N ckel N Cl	0 1 0 ?	35 5 3493	150	40
Boron H BO	0 1 0 5	5481 5440	150	50
Iron Fe(NO)	0 1 0 ?	3735 3737	00	50

The e e ult w e obtained with aqueou olutions of pu e single comp unds, with no th ions interfe ing

the wave length must be set very carefully This can be done by setting the wave length approximately with the instrument in operation and then turning it slightly to the right or left until the galvanometer shows maximum deflection

(5) The cap llary leading into the atomizer is very fine It has a tendency to become clogged gradually without the operator noticing it This results in gradually falling readings Therefore it is necessary to blow out the cap llary after every few determinations This can be done simply remove the stopper holding the capillary and with the air turned on hold a finger over the inside end for a few moments and replace Reve beratory sprayers of th s type were adopted for several r asons they will spray at continuous rates for a long time they require only small samples 5 ml in the present model they are economical of sample with a turnover of about 5 ml per m nute the residue of the sample is left unchanged in concentration and they are easy to clean and are chemically inert If the op erator desir s the present sprayers can be used for a continuous flow of sample attach drain tubes to the dra n plugs and trickle the sample directly to the sprayer

(6) The concentrat on of the element analyzed should be such that a straight line relationship will exist between C and ° T For the alkal metals this range is about 0 5 1 50 mEq/liter

c Theoretical Considerations Important for Accuracy

(1) *Wave Length* The wave length selected for each element depends on interfering substances Therefore although 556 mμ is the most intense calcium line it can not be u ed due to the intense sodium l ne at 5?8 Potassium is best read at 767 mμ

(2) *Spectral Line Interference* While the lines at which the three elements are read are quite widely separated and do not interfere with each other directly there are several indirect types of interference For example while the alkali metal radiation is of an atomic nature (and therefore quantized into discrete values) the calcium radiation is in large part of the molecular type This leads to a band spectrum of which the line at 422 mμ is merely the "band head" Therefore calcium is an interfering substance always to be taken into account Also while potassium and sodium separately have no radiation at each other's wave lengths each has a large effect in the presence of the other For example in a urine determination when the ratio of sodium to potassium is about 4 1 the reading of potassium at 767 mμ is increased considerably by the sodium present when read in a hot flame

(3) *Characteristics of the Flame* Since the calcium spectrum is of a molecular nature i e due to vibration and rotation it will increase in intensity with increasing flame temperature The hottest possible flame must be used in its determination However potassium radiates more strongly at a relatively low temperature due probably to a favorable tendency toward primary ionization at low temperature Moreover at low temperature sodium does not affect potassium to any measurable extent Sodium loses some of its intensity at low temperature but this is offset by the decreased interference due to any band spectra present The spraying of water reduces the luminosity of the flame as compared with a no spray flame doubtless due to lowering of the temperature Since water is always sprayed with a sample the back ground (no salt) must be read with a distilled water spray not just the bare flame With a spray of distilled water there is observed a continuous spectrum of rather constant intensity from 2500 Å to the deep red except for a hump near 3100 Å This hump is presumed to be a band spectrum perhaps of water since it rises with the addition of water to bare flame Resolved at narrow slits it shows two peaks at approximately 3067 Å and 3091 Å Its height appears to serve as a fiducial point for checking the adjustment of the flame from time to time Its intensity has not shown alteration with the spraying of a salt that gives no flame line in that region

Although we recommend a hot (gas oxygen) flame it is possible to use a cool (gas air) flame Under these conditions it is necessary to use wider slits and to sacrifice selective and sensitivity except in the case of potassium To increase luminosity a mirror of thick stainless steel has been mounted back of the flame This mirror helps when using a cool flame or a feeble radiating element

(4) *Standards* While any salts of sodium or potassium can be used in preparing standards this is not true of calcium for which the spectrum will vary with the anion used. In urinalysis the standard must be made up with the calcium in very nearly the same form as it occurs in urine and it is necessary to prepare a standard containing calcium phosphate chloride and sulfate

(5) *Concentrations* Samples should be of such a concentration of the ion under analysis that they fall into a range in which the C %T relation is linear For sodium and potassium this range is from 0 25 mEq/liter to about 1 5 mEq/liter For calcium however the linear range is at much higher concentrations so high that to obtain it and to get a measurable radiation the sample must be read undiluted This leads to difficulties due to differences in viscosity between the standards and sample and must be allowed for

(6) *Slit Width* Generally speaking a wide slit will increase interference possibilities Therefore the slit is made as small as is practicable For calcium a weakly radiating substance a slit of about 0 2 mm must be used to get a measurable response For potassium in a cool flame a slit of about 08 mm is used and for sodium in a moderately hot flame a slit of about 04 mm is used. With the Beckman DU Spectrophotometer the intensity of light received by the photo-

cells from a l ne is d rectly proport onal to the sl t width. E... ...
rece ved from a cont nuum is proport onal to the square of the ...
fore n p ck ng out l nes aga nst a cont nuous background it is ...
as narrow a sl t as is feas ble

H BALANCES

Standard textbooks cover very well the calibrat on ... analytical balances The torsion balance and damped balances ... in metabolic stud es that a brief discussion of them is included here

1 The Roller Smith Precision Torsion Balance Model B

a General Construction of the Instrument

In th s balance the ns de end of a sp ral spr ng is attached to the axis of ... the outs de end is attached to an index po nter (see Fig 9) Balances of ... max mum capac ty and sens tivity may be purchased. We have found th... for rap d accurate we gh ng of small amounts of material especial... sens t v ty is 0 2 per cent but the final accuracy of any we ght measurement ... on so many factors that it is imposs ble to say that the instrument ... nherent percentage accuracy W th the vern er the scale may be read to 1 ... scale d v s on

b Setting Up and Calibrating the Balance

(1) Set the nstrument on a table h gh enough so that the ope ato ... level w th the balance po nter It is a good dea to lay the balan... rying case
( ) Be certa n that the table s firm.
(3) Adjust the level ng screws unt l the bubble of the sp rit level ... center
(4) Release the beam by rotat ng the beam clamp lever unt l t po nts ward or back
(5) Adjust the ndex lever unt l the zero on the vern er coinc des ... zero on the d al
(6) The balance po nter should now co nc de w th its zero l ne If ... tate the zero adjuster knob on the back of the case until the bea...
(7) The balance s now ready to operate The ndex lever can be ... fe ent pos t ons by loosen ng the knurled nut
(8) The pan and compensat ng hook of the same we ght are selected.
(9) The balance s cal brated with the pan in place on the beam h... adjusted to zero w thout both the pan and compensatory h... standard we ght should be pu chased with each balance

c. Operating the Instrument

(1) *Before any we ght s placed on the hook the index lever is ro...* vern er s nearly to the h ghest value on the d al so that th... upper l m t of travel.
( ) Hang the material to be we ghed on the hook and rotate th... r ght unt l the beam drops down and the balance po nter ag... balance l ne
(3) The index vern er then ind cates the we ght d rectly on the d...

d. Disassembling the Roller Smith Balance

(1) Remove the front sp ndle knob tak ng off the index lever
( ) Remove the small plate on the left of the ca e which w... to be removed.

7 d h ll r ee ld ped ch are ns mg uto ad on f a 00 he e n h e nd num hang remo ed marks on t touch

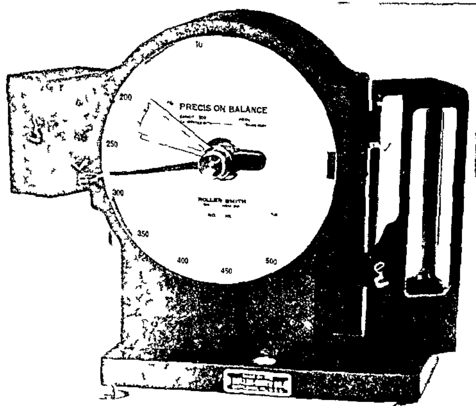


Fig 9 — The Roller Smith Preci ion Balance Model B (Reproduced with the permission of the manufacturers)

(3) The back plate screws should now be taken out and the mechanism removed from the rear taking care that the beam hooks are not injured when the mech anism is withdrawn from the case

(4) Jewels and pivots in the balance should never be oiled as oil at these points will in time collect dust and become gummy thus introducing friction into the bear ings The central knob which rotates the spindle should always be perfectly free to rotate and when an instrument is being overhauled it is a good plan to lubri cate this spindle which has a bearing in the cross arm The lubrication should be done with a little tallow

2 Damped Balances

a. General Principles

For the sake of speed without sacrifice of accuracy it is convenient to use damped balances either magnetic or air damped We prefer the air damped balance in which an inner and outer damping cyl nder are never in contact with each other and are affected only by air friction The action is so adjusted that the balance remains stationary after only one or two oscillations

b General Construction of the Sartorius Air Damped Balance

This balance has a multiple rider device for we ghts from one gram down to 10 mg For simplicity in reading an illuminated projection reader is switched on auto matically when the beam is released making values from 0 1 to 10 mg easy to read All parts of the reader are so arranged that opening of the doors has no effect on its adjustments Small errors in the zero point may be corrected by means of a special knob which is operated from the outside This balance has a capac ty of 00 gm with a sensitivity of 0 1 mg

c. Adjusting the Balance

(1) Level the case with the pillar on the bench by means of the leveling screws on the legs

( ) Place the fluted lock knob entirely into the hollow body bar in the middle of the plane and the two lock bolts into the holes made for this purpose These are on the left and right hand side of the pillar

(3) Switch off the lock knob and if the control bar is inside the pillar the lock mech anism rises and drops regularly

(4) Fit the beam on the center agate knife edge with the balance in the locked state and then check it

(5) Lock the balance again and fit the suspensions so that the holes are in f ont and side Then hang the pans in place

(6) On the right and left hand side of the pillar top are two very light aluminum bells that have two gaps at the bottom.

(7) Remove the bottom and put them into the outside cyl nder in order to hang them on the respective suspension hooks

(8) After hanging the inside cylinders shut the bells with the previously remove l bottoms but before fixing the pans it is advisable to make sure that the marks on each bell are in the right position

(9) Make sure when releasing the balance that the ins de cylinders do not touch the outside ones Then be certain that the balance rests at zero If not use the correction screws at either the left or r ght hand side of the beams

(10) The balance is then put in order by turning the knob outs de the case

(11) The annular riders for external mechanical appl cation of the weights are hung from the heaviest to the lightest that is from 00 to 10 mg on the doul le hook

d. Calibrating the Balance

(1) Before starting to use the balance set at zero by turning the sen itivity screws on the left or right hand side of the beam.

TABLE 2

TOLERANCES ESTABLISHED BY THE U S BUREAU OF STANDARDS FOR VOLUMETRIC APPARATUS

ITEM	PERMISSIBLE TOLERANCES (ML) FOR DIFFERENT SIZES															
	COMMON (E G 'EXAX')								BUREAU OF STANDARDS (E G NORMAX')							
PIPETTES	0 1 ml	0 2 ml	1 ml	– ml	5 ml	10 ml			0 1 ml	0 – ml	1 ml	– ml	5 ml	10 ml		
Ostwald	–	–	01[illegible]	012	02	04			–	–	–	006	01	0		
Volumetric	–	–	01[illegible]	012	02	04			–	–	–	006	01	02		
Serological	–	–	02	02	04	06			–	–	–	01	02	03		
Van Slyke Neill (for blood)	–	–	–	–	–	–			–	006	006	006	–	–		
Micro	–	–	–	–	–	–			001	002	–	–	–	–		
BURETTES	[illegible] ml	10 ml	[illegible]5 ml	50 ml	100 ml				5 ml	10 ml	5 ml	[illegible]0 ml	100 ml			
All types	0[illegible]	04	06	10	0				01	0	03	05	10			
VOLUMETRIC FLASKS	10 ml	25 ml	50 ml	100 ml	250 ml	500 ml	One l		10 ml	[illegible]5 ml	50 ml	100 ml	[illegible]50 ml	[illegible]00 ml	One l	
All types	06	06	10	16	24	30	60		03	03	05	08	12	15	30	
GRADUATED CYLINDERS	10 ml	[illegible]5 ml	50 ml	100 ml	[illegible]50 ml	500 ml	One l	Two l	10 ml	5 ml	50 ml	100 ml	[illegible]50 ml	500 ml	One l	Two l
All types	10	30	40	60	1 40	60	[illegible]0	10 0	08	14	0	3[illegible]	65	1 10	1 [illegible]0	3 50

( ) Place a 10 mg weight on the right pan release the balance and check the weight on the glass screen indicator It should read exactly 10 mg If not adjust the calibration screws until the reading is correct

(3) Then repeat the process by placing the weight on the left pan

(4) If the oscillations are too small raise the sens tivity screw in the center of the beam and if too violent lower the screw

(5) Be sure the pivot is not twisted otherwise the zero point will be off

e Operation of the Instrument

The operation is the same as for any analytical balance

I VOLUMETRIC ANALYSIS

Reference

Peffer E L and Mulligan G C U S Bureau of Standards Testing of Glass Volumetr c Apparatus Circular C434 Washington 1941 U S Government Printing Office

1 Definition of Volumetric Analysis

By volumetric analysis is meant quantitative analysis by the use of a measured amount of standard solutions The two aspects discussed below are first volumetric apparatus and second the preparation and standardization of common solutions

2 Volumetric Apparatus

a Pipettes

(1) *Common Pipettes* Many kinds of pipettes are used of which some of the more com mon kinds are illustrated in Fig 10 Calibrated pipettes differ in their accuracy the common commercial kind (e g Exax) having the widest tolerance and those calibrated to Bureau of Standards specifications the closest tolerance (e g Normax) Table . lists permitted tolerance for pipettes burettes volumetric flasks and graduated cylinders All pipettes are calibrated at 0 C and are marked either TD (to deliver) or TC (to contain) TD (to deliver) does not specify how delivery is to be made Hence there are two kinds of TD pipettes (a) pipettes with frosted tips must be blown out and (b) pipettes with no frosted band on the stem must be allowed to drain against the side of the vessel for the time specified on the pipette Bureau of Standards pipettes have been calibrated at the Bureau of Standards and carry individual calibra tion certificates Pipettes calibrated by the company (e g Normax) are guaranteed to fall with n Bureau of Standards tolerances When pipettes are marked TC (to contain) it is necessary either to use a nonwetting liquid such as mercury or to wash the pipette out pooling the wash fluid with the original sample

( ) *Syringe Pipettes* It is very conven ent to use syringe pipettes for sampling (See F g 11 Procurable from Macalaster Bicknell Co Cambridge Mass) These syringe pipettes were developed by Krogh and are calibrated in the same manner as ordinary pipettes. They can be adjusted to any volume from 0 1 ml to 10 0 ml For the analysis of food and feces we have found it convenient to calibrate a Luer Lok syringe because of the large bore at the tip of the syringe. The syringe makes the sampling much easier

b Burettes

(1) *Common Burettes* Burettes are procurable with different tolerances of accuracy (Table 2) but there is no real economy in purchasing other than those with the greatest accuracy (e g Normax) since few laboratories use many burettes.

(2) *Microburettes* When titrating small amounts of solution (as in serum potassium analysis when 0 05 ml of 0 1 N sodium thiosulfate is needed) we have found it convenient to use a Rehberg microburette (See Figs 1° 13 Procurable from Macalaster Bicknell Co, Cambridge, Mass) This is a capillary burette with a capacity of 0 1 or 0 2 ml and is graduated into fractions of 0 001 ml. At the lower end of the burette the capillary tubing bore becomes larger and is filled with mercury so that a turn of the screw causes the mercury to deliver aqueous

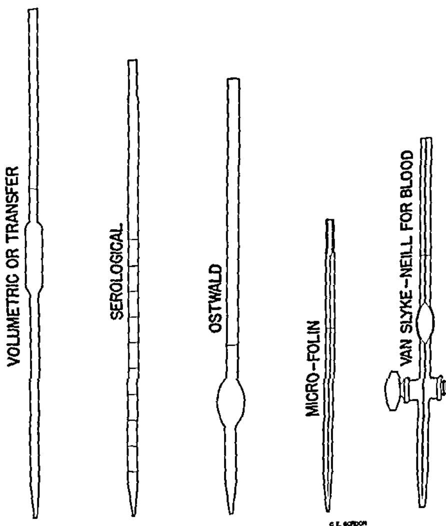


Fig 10—Common kind of volumetric pipettes.

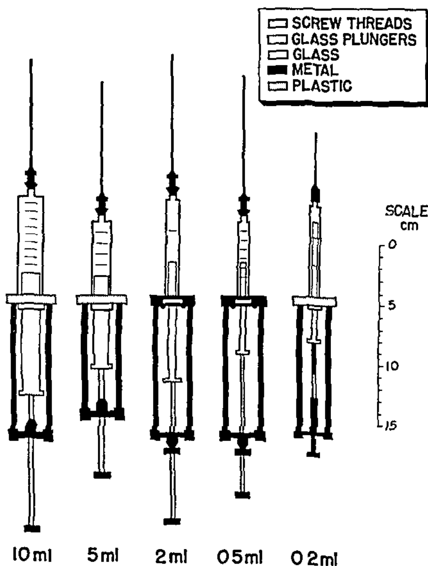
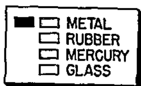
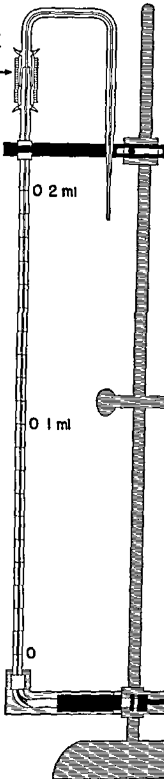
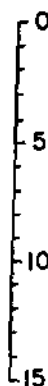

SCREW THREADS
GLASS PLUNGERS
GLASS
METAL
PLASTIC
SCALE
cm
0
5
10
15
10 ml
5 ml
2 ml
0 5 ml
0 2 ml
SYRINGE PIPETTES
C E GORDON

Fig 11

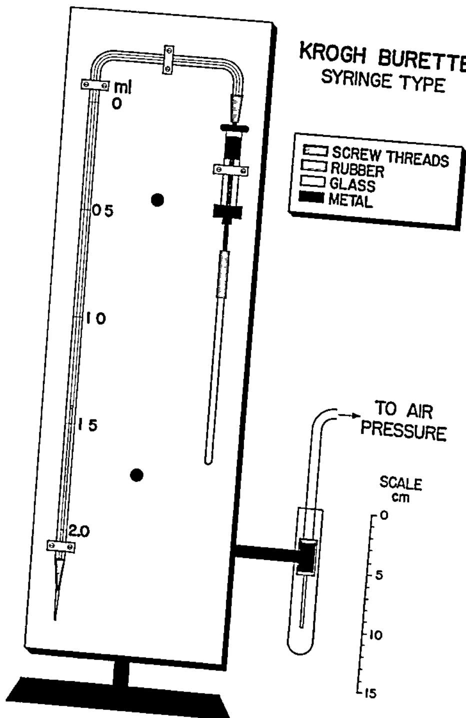






REHBERG MICRO BURETTE

Fig 12



KROGH BURETTE
SYRINGE TYPE
SCREW THREADS
RUBBER
GLASS
METAL
ml
0
0 5
1 0
1 5
2.0
TO AIR
PRESSURE
SCALE
cm
0
5
10
15
C. E. GORDON

Fig 12.

solutions with the greatest of accuracy The tip of the burette is on a greased swivel joint that makes titrating convenient

(3) *Syringe Screw Burette* This was popularized by Krogh (For an illustration see Fig 13) This burette has a capacity of 2 or 3 ml and is graduated to 0 01 ml. In titrations a twist of the screw delivers any fraction of a drop with great precision

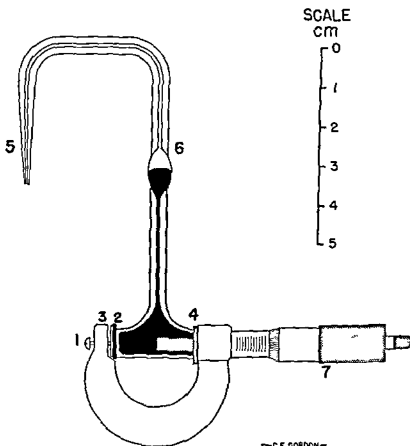


Fig 14 —Scholander's micrometer burette (Redrawn with permission of the author)

3 Calibration of Volumetric Apparatus

For most purposes volumetric apparatus is calibrated by weighing mercury or water delivered under the conditions of use of the apparatus At the same time the temperature of the fluid is measured and the *specific gravity* of the mercury or water is found in standard tables The *volume* delivered *then equals the weight of the fluid divided by the density at the given tem*

perature For quick and extremely accurate calibration of micro apparatus especially pipettes and burettes the Scholander micrometer burette is excel lent (Scholander P F Microburette' Science 95 177 178 Feb 1942 See Fig 14)

a. Principle of the Scholander Micrometer Burette

Mercury is displaced by the spindle of a machinist a micrometer The great precision of this kind of micrometer enables volumetric readings to be made with an accuracy of 0 0003 ml

b Construction of a Micrometer Burette (Fig 14)

(1) Procure a machinist a micrometer with jaws of capacity 1 to inches The best procurable micrometer should be purchased since only good ones have complete l nearity of scale

(2) *Replace the anv l with a set screw (#1 Fig 14)*

(3) The lower part of the burette is a cylindrical reservoir with a ground plane thick bottom and plane ground opening and an internal d ameter just enough to clear the spindle

(4) A fiber ring (#2) is placed on the closed end of the reservoir and next to it is placed a steel disk (#3) with a center punch to receive the set screw

(5) A fiber washer (#4) greased with a heavy stop-cock grease is placed at the *open end*

(6) Fasten the reservoir in the micrometer frame Mercury will not leak through the spindle bearing when it is properly greased

(7) If a small spindle is used the fiber washer (#4) must be reamed to a perfectly tight fit around the spindle which will then be proof against leakage of mercury

(8) The micrometer frame is clamped to a convenient stand and by movement of the sp ndle mercury is sucked in to fill the reservoir

(9) When the spindle is screwed out far enough to be level with the fiber washer air bubbles can be readily brought up into the funnel shaped opening to the capillary For proper functioning of the burette it is essential that the whole mercury con taining system be free of air The reservoir is air free when there is no visible bouncing when the mercury column is moved quickly from one place to another in the dry burette The last traces of air are removed by applying vacuum suc tion at the burette tip while the micrometer spindle is moved in and out

c Calibration of the Microburette

(1) Note that the precise size and shape of the glass part are immaterial. It is only the mercury expelled from the micrometer that is actually measured.

( ) Bring the micrometer clo e to 10 making sure that mercury fills the burette exactly to the tip *Read.*

(3) Into a we ghed vessel force mercury until the micrometer reads close to 0 Read again

(4) Measure the temperature of the burette by laying a thermometer alongside

(5) Reweigh the vessel

(6) From standard tables find the density of mercury at the observed micrometer temperature

(7) *Compute the calibration constant of the burette from the equation*

$$\text{Milliliters per scale divis on} = \frac{(\text{mg Hg})}{(\text{den ity } Hg)} \div (\text{Read ng 1 minus Reading 2})$$

(8) For greatest accuracy recalibrate by successive tenths of an inch with weighings each time.

d Calibration of Apparatus With the Scholander Micrometer Burette

(1) Note that for calibration of other apparatus the temperature of the micrometer and of the apparatus calibrated must be the same but that the absolute temperature is immaterial This is so because the initial calibration of the micrometer burette gives calibration constant equating scale divisions of the micrometer with volume

(2) As an example let us assume that a serological pipette of 0 1 ml capacity is to be calibrated

(a) Place tightly on the tip of the burette a piece of strong capillary rubber tubing extending about 2 cm beyond the burette tip
(b) Make sure that the micrometer is filled almost to 10
(c) Insert firmly the mouth end of the pipette and clamp it almost horizontal, with the tip end slightly higher than the mouth end (If the pipette is clamped upright subsequent pressure of the mercury column might give erroneous readings or even spillage)
(d) Manipulate micrometer until mercury column is exactly at top mark of pipette
(e) Read micrometer
(f) Force mercury column exactly to tip of pipette
(g) Read micrometer again
(h) Calculate volume of pipette as follows

ml in pipette = (Reading 1 minus Reading 2) × (Micrometer calibration)

4 Standard Solutions

a. Definitions

(1) The *atomic weight* of an element is the relative weight of its atom compared to the common isotype of oxygen as 16

(2) The *molecular weight* of a compound is the sum of the atomic weights of all the atoms composing the molecule

(3) The *equivalent weight* or *combining weight* of an element or compound is that weight which will react with or release 8 units of oxygen or any equivalent thereof Equivalent weight depends upon the specific reaction under consideration.
(a) The *gram equivalent* is the equivalent weight in grams
(b) The *milliequivalent (mEq)* is the equivalent weight in milligrams

(4) A *molar solution* contains one gram molecular weight in 1 liter e g a molar solution of sulfuric acid contains 98 08 gm of sulfuric acid diluted to a liter of solution

(5) A *normal solution* contains one gram equivalent weight of a substance in a liter of solution The gram equivalent weight depends upon the reaction for which the substance is used E g a normal solution of sulfuric acid will contain $\frac{98\ 08}{2} = 49\ 04$ gm of the acid diluted to one liter with water

(6) A *normal reducing solution* contains in one liter of solution one gram of oxidizable hydrogen or its equivalent in other reducing substances Oxalic acid (H C_2O 2H O) contains two hydrogen atoms oxidizable by pota sium permanganate (KMnO) so that its normal reducing solution contains $\frac{1\ 6}{2} = 63$ gm per liter

(7) One liter of a *normal oxidizing solution* will oxidize one gram atom of hydrogen or equivalent reducing substance A normal solution of KMnO for use in the reaction with oxalic acid contains $\frac{158\ 03}{5} = 31\ 606$ gm per liter

5 Preparations of Standard Solutions

a. Hydrochloric Acid HCl, mol. wt. 36 47

(1) Preparation of 1 000 N Solution Mix 500 ml of water and 500 ml of concentrated hydrochloric acid (sp gr 1) At 25 C adjust density to 1 096 with more acid or water as needed Distill at the rate of 3 4 ml/minute and collect the last quarter of the distillate for use Record the barometric pressure during distillation Prepare exactly 1 000 N HCl by use of the following table

Barometric Pressure mm Hg	HCl Concentration % by weight	Required for One Liter 1 000 N HCl gm
730	0 93	179 56
740	0 69	179 77
7 0	0 4.	179 98
760	0 1	180 19
770	0 197	180 41

Prepare standard hydrochloric acid by diluting the proper weight of HCl to volume E g Barometric pressure is 760 To prepare exactly 1 000 N HCl weigh out exactly 180 19 gm of distillate and dilute to 1 liter with water

(2) Standardization This is primary standard and needs no standardization

b Potassium Hydrogen Phthalate $C_6H_4(COOH)COOK$, mol. wt 204 14

(1) Preparation of 0 1000 N Solution Obtain pure dry salt from the U S Bureau of Standards Dilute 0 414 gm to 1 liter with water When titrating with this solution use phenolphthalein for indicator Before the end point is reached boil the aliquot being titrated so as to remove dissolved CO_2

(2) Standardization This is a primary standard and needs no standardization

c Sodium Hydroxide NaOH, mol. wt 40 01

(1) Stock Concentrated Solution For many purposes it is desirable to keep on hand a concentrated solution In a 500 ml Pyrex flask or bottle mix approximately 00 gm NaOH and 00 ml water Cool and stopper with a rubber not glass stopper This keeps indefinitely but should not be exposed to air except as necessary Carbonates are practically insoluble at this concentration and will settle out. The supernatant fluid is approximately 1 5 N in NaOH

(2) Preparation of 1 000 N Sodium Hydroxide Boil approximately 1400 ml of distilled water to remove CO_2 and cool to room temperature Decant or filter through asbestos 60 0 ml of concentrated stock solution Dilute 50 ml of this to exactly one liter with boiled distilled water

(3) Standardization Using phenolphthalein as indicator and standard potassium acid phthalate in the burette pipette 20 ml portions of sodium hydroxide into 50 ml Erlenmeyer flasks. Add approximately 15 ml of the standard acid bring to boil and titrate to end point. If alkali is over 1 000 N add water according to the following equation

To 1000 ml of NaOH add (1000) × (actual normality − 1 000)

Repeat standardization until exactly 1 000 N is reached. Store in Pyrex with rubber not glass stopper Avoid exposure to air

d. Oxalic Acid, $C_2H_2O_4 \cdot 2H_2O$ mol wt. 126.06

(1) Preparation of 0 1000 N Solution Dissolve exactly 6 303 gm of pure dry oxalic acid in water and dilute to 1 liter This keeps indefinitely

( ) Standardization Titrate against hot sodium hydroxide with phenolphthalein as indicator

d. Calibration of Apparatus With the Scholander Micrometer Burette

(1) Note that for calibration of other apparatus the temperature of the micrometer and of the apparatus calibrated must be the same, but that the absolute temperature is immaterial This is so because the initial calibration of the micrometer burette gives calibration *constant equating scale divisions of the micrometer with* volume

(2) As an example let us assume that a serological pipette of 0 1 ml capacity is to be calibrated.

(a) Place tightly on the tip of the burette a piece of strong capillary rubber *tubing extending about 2 cm beyond the burette tip*

(b) Make sure that the micrometer is filled almost to 10

(c) Insert firmly the mouth end of the pipette and clamp it almost horizontal, with the tip end slightly higher than the mouth end (If the pipette is clamped upright subsequent pressure of the mercury column might give erroneous readings or even spillage)

(d) Manipulate micrometer until mercury column is exactly at top mark of pipette

(e) Read micrometer

(f) Force mercury column exactly to tip of pipette

(g) Read micrometer again

(h) Calculate volume of pipette as follows

ml in pipette = (Reading 1 minus Reading 2) × (Micrometer calibration)

4 Standard Solutions

a. Definitions

(1) The *atomic weight* of an element is the relative weight of its atom compared to the common isotype of oxygen as 16

(2) *The molecular weight of a compound is the sum of the atomic weights* of all the atoms composing the molecule

(3) The *equivalent weight* or *combining weight* of an element or compound is that weight which will react with or release 8 units of oxygen or any equivalent thereof Equivalent weight depends upon the specific reaction under consideration

(a) The *gram equivalent* is the equivalent weight in grams

(b) The *milliequivalent (mEq)* is the equivalent weight in milligrams

(4) A *molar solution* contains one gram molecular weight in 1 liter e g a molar solution of sulfuric acid contains 98 08 gm of sulfuric acid diluted to a liter of solution

(5) A *normal solution* contains one gram equivalent weight of a substance in a liter of solution The gram equivalent weight depends upon the reaction for which the substance is used E g a normal solution of sulfuric acid will contain $\frac{98\ 08}{2} = 49\ 04$ gm of the acid diluted to one liter with water

(6) A *normal reducing solution* contains in one liter of solution one gram of oxidizable hydrogen or its equivalent in other reducing substances Oxalic acid (H C_2O H O) contains two hydrogen *atoms oxidizable by potassium permanganate* (KMnO) so that its normal reducing solution contains $\frac{1\text{-}6}{2} = 63$ gm per liter

(7) One liter of a *normal oxidizing solution* will oxidize one gram atom of hydrogen or equivalent reducing substance A normal solution of KMnO for use in the reaction with oxalic acid contains $\frac{158\ 03}{5} = 31\ 606$ *gm per liter*

5 Preparations of Standard Solutions

a. Hydrochloric Acid HCl, mol wt. 36 47

(1) Preparation of 1 000 N Solution Mix 500 ml of water and 500 ml of concentrated hydrochloric acid (sp gr 1) At ␣ C adjust density to 1 096 with more acid or water as needed Distill at the rate of 3 4 ml/minute and collect the last quarter of the distillate for use Record the barometric pressure during distillation Prepare exactly 1 000 N HCl by use of the following table

Barometric Pressure mm Hg	HCl Concentration % by weight	Required for One Liter 1 000 N HCl gm
730	0 93	179 56
740	0 69	179 77
750	0 45	179 98
760	0 1	180 19
770	20 197	180 41

Prepare standard hydrochloric acid by diluting the proper weight of HCl to volume E g Barometric pressure is 760 To prepare exactly 1 000 N HCl weigh out exactly 180 19 gm of distillate and dilute to 1 liter with water

(2) Standardization This is primary standard and needs no standardization

b Potassium Hydrogen Phthalate $C_6H_4(COOH)COOK$, mol. wt. 204 14

(1) Preparation of 0 1000 N Solution Obtain pure dry salt from the U S Bureau of Standards Dilute 0 414 gm to 1 liter with water When titrating with this solution use phenophthalein for indicator Before the end point is reached boil the aliquot being titrated so as to remove dissolved CO_2

( ) Standardization This is a primary standard and needs no standardization.

c Sodium Hydroxide NaOH, mol. wt. 40 01

(1) Stock Concentrated Solution For many purposes it is desirable to keep on hand a concentrated solution. In a 500 ml Pyrex flask or bottle mix approximately 200 gm NaOH and 00 ml water Cool and stopper with a rubber not glass stopper This keeps indefinitely but should not be exposed to air except as necessary Carbonates are practically insoluble at this concentration and will settle out. The supernatant fluid is approximately 17 N in NaOH

(2) Preparation of 1 000 N Sodium Hydroxide Boil approximately 1400 ml of distilled water to remove CO_2 and cool to room temperature Decant or filter through asbestos 60 70 ml of concentrated stock solution Dilute 59 ml of this to exactly one liter with boiled distilled water

(3) Standardization Using phenolphthalein as indicator and standard potassium acid phthalate in the burette pipette 0 ml portions of sodium hydroxide into 50 ml Erlenmeyer flasks. Add approximately 15 ml of the standard acid bring to boil and titrate to end point If alkali is over 1 000 N add water according to the following equation

To 1000 ml of NaOH add (1000) × (actual normality − 1 000)

Rep at standardization until exactly 1 000 N is reached. Store in Pyrex with rubber not glass stopper Avoid exposure to air

d. Oxalic Acid, $C_2H_2O_4 \cdot 2H_2O$ mol wt. 126.06

(1) Preparation of 0 1000 N Solution Dissolve exactly 6 303 gm of pure dry oxalic acid in water and dilute to 1 liter This keeps indefinitely

(2) Standardization Titrate against hot sodium hydroxide with phenolphthalein as indicator

e Sulfuric Acid, H_2SO_4, mol. wt. 98 08

(1) Preparation of 1 N Solution Add about 500 ml water to a 1 liter volumetric flask Add approximately 29 ml of concentration sulfuric acid *(sp gr 1.84)* Dilute to 1 liter with water

(2) Standardization Titrate against standard sodium hydroxide The concentration should be near 1 N

f Benzoic Acid C_6H_5COOH mol wt 122 05

(1) Preparation of 0 1000 N Solution Weigh out exactly 12 205 gm dry acid into a one liter volumetric flask Add about 500 ml warm 95% ethyl alcohol, dissolve and dilute to 1 liter

(2) Standardization Titrate against hot sodium hydroxide using phenolphthalein as indicator

g Silver Nitrate $AgNO_3$ mol wt. 169 89

(1) Preparation of 0 1000 N Solution Dissolve 16 989 gm pure fused silver nitrate in water add about 100 ml of concentrated nitric acid and dilute to 1 liter Keep *in the dark in a brown bottle*

(2) Standardization This may be used as a primary standard without standardization However if standardization is desired a gravimetric procedure is recommended as follows

(a) *Make an approximately 1% solution of HCl by diluting 100 ml of con*centrated HCl with 900 ml of water

(b) Running triplicates prepare 250 ml beakers with exactly 50 ml of silver nitrate solution

(c) With stirring add 75 ml of HCl small portions at a time

(d) Bring to boil and allow to cool and settle in the dark

(e) Saving all washings filter through weighed sintered glass filter Wash with 5 successive 50 ml portions of water containing a few drops of nitric acid and test the last washing with silver nitrate for presence of chloride If negative wash with two 50 ml portions of plain water

(f) Record volume and temperature of total washings

(g) *Dry precipitate to constant weight at 110 C*

(h) Calculation

$$\text{Normality of } AgNO_3 = \frac{(\text{gm ppt}) + \left(\frac{\text{total ml washings}}{1000} \times \text{loss/liter}\right)}{\left(\frac{\text{ml } AgNO_3 \text{ used}}{1000}\right) \times (143\ 34)}$$

(i) Solubility of AgCl in 1% HCl is
0 000 gm AgCl/1 at 20 C and
0 0027 gm AgCl/1 at 30 C

h Potassium Thiocyanate KCNS mol. wt 97 16

(1) Preparation of 0 10 N Solution Dissolve 9 8 gm KCNS in water and dilute to 1 liter

(2) Standardization To 20 ml of 0 1000 N $AgNO_3$ add 5 ml concentrated HNO_3 25 ml of water and 1 ml of saturated aqueous ferric alum solution Titrate with KCNS to the first permanent *salmon pink color*

i Sodium Oxalate $Na_2C_2O_4$, mol. wt. 133 99

(1) Preparation of 0 1000 N Solution Weigh out exactly 6 700 gm of pure dry salt (Bureau of Standards) Dissolve and dilute to 1 liter with water

(2) Standardization This is a primary standard and requires no standardization.

j Potassium Permanganate $KMnO_4$, mol. wt. 158 03

(1) Preparation of 0 1 N Solution Dissolve approximately 3 3 gm pure KMnO in 1 liter of water This must age Let stand in dark several months and filter through asbestos

( ) *Standardization To* 20 *ml of* 0 1000 *N sodium oxalate in a* 500 *ml Erlenmeyer* flask add 00 ml of water and 3 ml of concentrated H_2SO_4. Heat to 0 C and titrate to first pink persisting 30 seconds Be sure to run adequate numbers of blanks

k. Potassium Iodate KIO_3, mol wt. 214 03

(1) Preparat on of 0 1000 N Solution Weigh out exactly 3.56 gm pure dry KIO dissolve in water and dilute to 1 liter

(2) Standardization This is a primary standard and need no standardization

l. Potassium Dichromate $K_2Cr_2O_7$ mol wt. 294 22

(1) Preparation of 0 1000 N Solution Dissolve exactly 4 904 gm of pure salt in water and dilute to exactly 1 liter

( ) Standardizat on This may be used as a primary standard

m. *Sodium Thiosulfate* $Na_2S_2O_3$ $5H_2O$ *mol wt* 248 19

(1) Preparation of 0 1000 N Solution Dissolve 24 819 gm of pure salt in approximately 500 ml water and add 1 ml of approximately 1 N NaOH Dilute to 1 liter with water

(2) Standardizat on against *Iodate* To 5 ml of standard 0 1000 N iodate in an Erlenmeyer flask add 10 ml of 10% KI and 0 ml of 1 N H_2SO_4 or HCl Titrate with thiosulfate until the solut on is pale yellow Add a few drops of aqueous starch solution (1%) and titrate to disappearance of blue color

(3) Standardization against Permanganate Dissolve approximately 1 gm of KI in 10 ml of water add approximately 3 ml of concentrated HCl and pipette exactly 5 ml of 0 1000 N KMnO Titrate with thiosulfate until bright yellow then add ½ to 1 ml of 1% starch and titrate to disappearance of the blue color

(4) Standardization with Dichromate In a 00 ml Erlenmeyer flask mix approximately 300 ml of water 5 gm of KI 4 gm of $NaHCO_3$ and then excess of concentrated HCl Add exactly 25 ml of 0 1000 potassium dichromate and let stand in the dark 10 minutes Titrate with thiosulfate using ½ to 1 ml of 1 starch as indicator

n. Iodine I_2 mol wt 126 92

(1) Preparation of 0 1 N Solution In approximately 0 0 ml of water dissolve 30 gm of pure potassium iodide and 13 gm of iodine (resublimed) Dilute with water to 1 liter

(2) Standardization In a 100 ml Erlenmeyer flask mix exactly 0 ml of iodine and approximately 50 ml of water Titrate with 0 1000 N thiosulfate to faint yellow add approximately 1 ml of 1% starch solution and titrate to disappearance of blue color

J INSTRUMENTATION MISCELLANEOUS REFERENCES

Balance

Lowry O H A Simple Quartz Torsion Balance J Biol Chem 152 295 (Feb) 1944

Burette

Longwell B and Hill R M A Modified Rehberg Burette for Use With Titrating Solutions Which React With Mercury J Biol. Chem 112 313 (Dec) 19

Scholander P F Edwards G A and Irving L Improved Micrometer Burette J Biol Chem 148 495 500 (June) 1943

Scholander P F Microburette, Science 95 177 8 (Feb) 194

Rehberg P B A Method of Microtitration Biochem J 19 ( ) 0 (Feb) 1

Electron Microscope

Wyckoff, R W G Electron Microscopy Technique and Applications New York 1949 Interscience Publishers, p 248

Electrophoresis

Abramson H A Moyer L S and Gorin M H Electrophoresis of Proteins New York 1942 Reinhold Publishing Corp

Alberty R A An Introduction to Electrophoresis I Methods and Calculations J Chem Ed 25 426 433 (Aug) 1948

Blix G and Pedersen K O Electrophoresis and Ultracentrifugation of Lipide Free Human Serum Acta Chem Scandinav 1 (5) 511 520 1947

Jager B V Schwartz, T B Smith E L, Nickerson M and Brown D M Comparative Electrophoretic and Chemical Estimation of Human Serum Albumin an Evaluation of Six Methods J Lab & Clin Med 35 76 86 (Jan) 1950

Labhart H and Staub H Mikro Elektrophorese, Helv Chim Acta 30 ( ) 1954 1964 1947

Lang E R Van Winkle Q and France W G Electrophoresis in an Ultracentrifugal Field J Colloid Sci 2 315 332 (June) 1947

Longsworth L G A Modification of the Schlieren Method for Use in Electrophoretic Analysis J Am Chem Soc 61 529 530 (Feb) 1939

Longsworth L G The Quantitative Interpretation of the Electrophoretic Pattern of Proteins J Physical & Colloid Chem 51 171 183 (Jan) 1947

Moore D H and White J U A New Compact Tiselius Electrophoresis Apparatus Rev Sci Instr 19 700 706 (Oct) 1948

Popjak G and McCarthy, E F Osmotic Pressure of Experimental and Human Lipaemic Sera Evaluation of Albumin Globulin Ratios With the Aid of Electrophoresis Biochem J 40 789 803 1946

Stern K G Aminco Stern Electrophoresis Apparatus American Instrument Company Silver Springs Md Bull No 2175 (June) 1949

Flame Photometer

Domingo W R, and Klyne W Photoelectric Flame Photometer Biochem J 45 400 408 1949

Gas Analysis

Scholander P F and Evans H J Microanalysis of Fractions of a Cubic Millimeter of Gas J Biol Chem 169 (3) 551 560 (Aug) 1947

Scholander P F and Irving L Micro Blood Gas Analysis in Fractions of a Cubic Millimeter of Blood J Biol Chem 169 (3) 561 569 (Aug) 1947

Infrared Spectrometer

Gore R C Infrared Spectrometry of Small Samples With the Reflecting Microscope Science 110 710 711 (Dec) 1949

Manometric Techniques

Dixon M Manometric Methods as Applied to the Measurement of *Cell Respiration and* Other Processes ed 2 London 1943 Cambridge University Press

Holmes E The Metabolism of Living Tissues New York 1937, The Macmillan Company

Laser H and Rothschild L A New Manometric Method for Determination of *Respiratory* Quotients Biochem J 45 598 612 1949

Umbreit W W Burris R T and Stauffer J F *Manometric Techniques* and Related Methods for the Study of Tissue Metabolism 1st ed., *4th Printing Minneapolis* (Feb) 1948 Burgess Publishing Company

Mass Spectrometer

Hipple J A and Shephard M Mass Spectrometry Anal Chem 21 3 36 (Jan.) 1949

Kerwin L New Type Mass Spectrometer Rev Scient Instruments 21 96 97 (Jan) 1950

Nier A O Mass Spectrometer Rev Scient Instruments 18 398 1947

Washburn H. W Mass Spectrometer U S Nav Bull Suppl (March April) 1948. 51

Wiley H F A Mass Spectrometer for Isotope Ratio Determinations Science 110 355 (Oct) 1949

Microrespirometer

Scholander P F *Volumetric Microrespirometers* Rev Sc Instr 13 3 33 (Jan) 194

Oximeter

Millikan G A *The Oximeter* an Instrument for Measuring Continuously the *Oxygen* Saturation of Arterial Blood in Man Rev Scient Instruments 13 (10) 434 444 *(Oct)* 1942

Hemingway A and Taylor C. B Laboratory Tests of the Oximeter With Automatic Compensation for Vasomotor Changes J Lab & Clin. Med. 29 987 991 (Sept) 1944
Comroe J H Jr., and Walker P Normal Human Arterial Oxygen Saturation Determined by Equilibration With 100% O in Vivo and by the Oximeter Am J Physiol 152 365 371 (Feb) 1948

Pipettes

Shohl A. T A Pipet for Micro analyses J Am Chem. Soc 60 417 (Feb) 19 8

Polarography

Kolthoff I M and Lingane J J Polarography (Revised Reprint), New York 1946 Interscience Publishers

Respirometer

Scholander P F Volumetric Plastic Micro Respirometer, Rev Scient Instruments 21 378 380 (Apr) 1950

Ultracentrifuge

Pedersen K O Ultracentrifugal Studies on Serum and Serum Fractions Upsala Sweden 1945, Almquist and Wiksells
Pickels E G The Ultracentrifuge Practical Aspects of the Ultracentrifugal Analysis of Proteins Chem Rev 30 341 355 (June) 1942
Svedberg T., and Pedersen K O The Ultracentrifuge, London 1940 Oxford University Press

Ultrafiltration

Lavietes P H Anaerobic Ultrafiltration J Biol Chem 1 0 26 45 (Aug) 1937

Ultramicroanalysis

Kirk P L Quantitative Ultramicroanalysis, New York 1950 John Wiley & Sons Inc.

Electron Microscope

Wyckoff R W G Electron Microscopy Technique and Applications, New York, 1949 *Interscience Publishers* p 248

Electrophoresis

Abramson H A Moyer L S and Gorin M H Electrophoresis of Proteins New York 1942 Reinhold Publishing Corp

Alberty R A An Introduction to Electrophoresis I Methods and Calculations J Chem Ed 25 426-433 (Aug) 1948

Blix G and Pedersen K O Electrophoresis and Ultracentrifugation of Lipide Free Human Serum Acta Chem Scandinav 1 (5) 511 520 1947

Jager B V Schwartz T B Smith E L, Nickerson M and Brown D M Comparative Electrophoretic and Chemical Estimation of Human Serum Albumin an Evaluation of Six Methods J Lab & Clin Med 35 76 86 (Jan) 1950

Labhart H, and Staub H Mikro Elektrophorese, Helv Chim Acta 30 (7) 1954 1961 1947

Lang E P Van Winkle Q and France W G Electrophoresis in an Ultracentrifugal Field J Colloid Sci 2 315 329 (June) 1947

Longsworth L G A Modification of the Schlieren Method for Use in Electrophoretic Analysis J Am Chem Soc 61 529 530 (Feb) 1939

Longsworth L G The Quantitative Interpretation of the Electrophoretic Pattern of Proteins J Physical & Colloid Chem 51 171 183 (Jan) 1947

Moore D H, and White J U A New Compact Tiselius Electrophoresis Apparatus Rev Sci Instr 19 700 706 (Oct) 1948

Popjak G and McCarthy E F Osmotic Pressure of Experimental and Human Lipaemic Sera Evaluation of Albumin Globulin Ratios With the Aid of Electrophoresis, Biochem J 40 789 803 1946

Stern K G Aminco Stern Electrophoresis Apparatus American Instrument Company Silver Springs Md, Bull No 2175 (June) 1949

Flame Photometer

Domingo, W R and Klyne W Photoelectric Flame Photometer Biochem J 45 400 408 1949

Gas Analysis

Scholander P F and Evans H J Microanalysis of Fractions of a Cubic Millimeter of Gas J Biol Chem 169 (3) 551 560 (Aug) 1947

Scholander, P F and Irving L Micro Blood Gas Analysis in Fractions of a Cubic Millimeter of Blood, J Biol Chem 169 (3) 561 569 (Aug) 1947

Infrared Spectrometer

Gore, R C Infrared Spectrometry of Small Samples With the Reflecting Microscope Science 110 710 711 (Dec) 1949

Manometric Techniques

Dixon M Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processes ed 2 London 1943, Cambridge University Press

Holmes E The Metabolism of Living Tissues New York 1937 The Macmillan Company

Laser, H and Rothschild L A New Manometric Method for Determination of Respiratory Quotients Biochem J 45 598 612 1949

Umbreit, W W Burris R T, and Stauffer J F Manometric Techniques and Related Methods for the Study of Tissue Metabolism 1st ed, 4th Printing, Minneapolis, (Feb) 1949 Burgess Publishing Company

Mass Spectrometer

Hipple J A and Shephard M Mass Spectrometry Anal Chem 21 32 36 (Jan) 1949

Kerwin L New Type Mass Spectrometer Rev Scient Instruments 21 96 97 (Jan) 1950

Nier A O Mass Spectrometer Rev Scient Instruments 18 398 1947

Washburn H W Mass Spectrometer, U S Nav Bull Suppl (March April) 1948

Wiley, H F A Mass Spectrometer for Isotope Ratio Determination Science 110 354 355 (Oct) 1949

Microrespirometer

Scholander P F Volumetric Microrespirometers Rev Sci Instr 13 32 33 (Jan) 1942

Oximeter

Millikan G A. The Oximeter an Instrument for Measuring Continuously the Oxygen Saturation of Arterial Blood in Man Rev Scient Instruments 13 (10) 434-444 (Oct) 1942

Hemingway A., and Taylor C B Laboratory Tests of the *Oximeter* With Automatic Compensation for Vasomotor Changes J Lab & Clin. Med. 29 987 991 (Sept) 1944
Comroe, J H Jr, and Walker P Normal Human *Arterial* Oxygen Saturation *Determined* by Equilibration With 100% O in Vivo and by the Oximet r Am J Physiol 152 365 371 (Feb) 1948

Pipettes
Shohl A. T A Pipet for *Micro* analyses J Am. Chem. Soc 60 417 (Feb) 19 8

Polarography
Kolthoff I M and Lingane J J Polarography (*Revised* E print), New York 1946, Interscience *Publishers*

Respirometer
Scholander P F Volumetric Plastic Micro Respiromete Rev Scient Instruments 21 78 3 0 (Apr) 1950

Ultracentrifuge
Pedersen K O *Ultracentrifugal* Studi s on Serum and *Serum* Fractions Upsala Sweden, 1945 Almquist and *Wiksells*
Pickels E G The Ultracentrifuge Practical *Aspects* of the *Ultracentrifugal* Analys s of *Proteins* Chem Rev 30 341 355 (June) 194
Svedberg T and Pedersen K O The Ultracentrifuge *London* 1940 Oxford University *Press*

Ultrafiltration
Lavietes P H Anaerobic Ultrafiltration J Biol Chem 120 267 275 (Aug) 1937

Ultramicroanalysis
Kirk P L Quantitative Ultramicroanalysis *New* York 1950 John Wiley & Sons In

SECTION IV

BIOCHEMICAL PROCEDURES

A MINERALS

1 Total Water and Total Solids in Food, Tissue, Cells, Serum and Feces

Reference

Association of Official Agricultural Chemists "Official and Tentative Methods of Analysis Total Solids Method I Official" ed. 5, Washington D C 1940 p ?12

Principle

The difference in weight between a sample *before* and *after drying* at 110 C over night is the amount of water in the sample The residue represents the total solids of the sample

Apparatus

1 A number of weighing bottles with lids
2 An analytical balance
3 An oven maintained at 110 C
4 A desiccator

Reagents

None

Procedure

1 Weigh accurately a dry weighing bottle that has been cooled in a desiccator for at least one half hour
2 Add the sample and weigh again.
3 Open the dish and leave it in a 110 C oven overnight
4 Cool in the desiccator clo e and weigh again.

Calculation

1 $\text{gm water \%} = \dfrac{\text{(Weight of bottle before drying} - \text{weight of bottle after)}}{\text{gm sample taken}} \times 100$

2 $\text{gm solids \%} = \dfrac{\text{(Weight of bottle after drying} - \text{initial weight of bottle)}}{\text{gm sample taken}} \times 100$

Example

Weight of weighing bottle	10 9586 gm
Weight with sample of muscle	12 5542 gm
Weight after drying	*11.3507 gm*
Therefore, weight of sample	1 5956 gm
weight of residue	0 3921 gm
and weight of water	1.2035 gm

$$\text{gm water \%} = \frac{1\ 2035}{1\ 5956} \times 100 = 75\ 4$$

$$\text{gm solids \%} = \frac{0\ 3921}{1\ 5956} \times 100 = 24\ 6$$

Precautions

1 In the presence of unsaturated fat in large concentration the weight of the residue of a specimen sometimes reaches a minimum and then rises again as oxygen is absorbed. It is desirable to make weighings as often as every six hours to determine the minimum

2 In the pre ence of a high concentration of water preliminary drying on a steam bath is necessary to prevent frothing which often occurs when a sample is placed directly into an oven

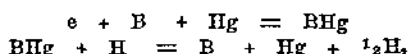
2 Total Base in Serum, Urine Cells and Tissue

Reference

Consolazio W V and Talbott J H The Determ nation of Total Base in Biological Mater al by Electrodialysis J Biol Chem. 132 753 67 (Feb) 1940

Principle

The basic ions are electrodialysed into mercury with which they amalgamate The amalgam is decomposed w th standard acid and the excess acid back titrated with standard alkali



$$e + B + Hg = BHg$$
$$BHg + H = B + Hg + \frac{1}{2}H_2$$

Apparatus

1 Total base mach ne with accessories (Macalaster Bicknell Company Cambr dge Massachusetts An indiv dual unit is illustrated in Fig 15) This includes two milliammeters a pilot lamp a rheostat a shunt and a switch It is operated at 110 or 2 0 volts DC the possibility of using lower voltages has not been tested fully It draws 65 milliamperes at full load. The anode is a platinum electrode fused in a Pyrex cup and connected by a metal socket directly to the source of power The anode acid conductor of Adair and Keys (Adair G S and Keys A B J Physiol 81 16 1934) has been eliminated

Anode cups should not be cleansed with cleaning solution Precipitat on of chromium on platinum may introduce serious errors It is recommended that the cups be brushed with water filled with concentrated nitric acid and al lowed to stand for several hours Several rinses with distilled water remove all adherent base

2 #300 plain transparent cellophane (Dupont)

3 Rehberg microburette 0 ml

4 Slow speed otating motor for applying collodion

5 Membrane tubes These are prepared as follows Cut cellophane into 3 nch squares and soak for 5 minutes in dist lled water Apply the wet squares to the open end and opposite the constriction Now with the thumb and the forefinger pull the wet membrane taut by applying pressure along the tube wall and away from the former opening The membrane should always be stretched across the grain s nce this axis has greatest elasticity A good proce lure to follow is to stretch the membrane slightly cross grain before applying it to the membrane tube Allow the membrane to dry 1 ? hours The excess cellophane is now trimmed with a sharp razor or scalpel about ¼ inch down the tube wall The partially made membrane tubes are now rotated parallel to the floor about 20 30 r p m and while they are rotating a thin coat of collodion is applied Starting at the edge of the tube pour a continuous stream about ¼ inch from the tube end The collodion sets in a minute or two Care should be taken that collodion does not flow over the membrane face After drying (3 5 hours) the finished membrane tubes are rinsed thoroughly with distilled water and stored in dis tilled water in the refrigerator Membrane tubes prepared in this fashion have

ELECTRODIALYSIS UNIT FOR TOTAL BASE

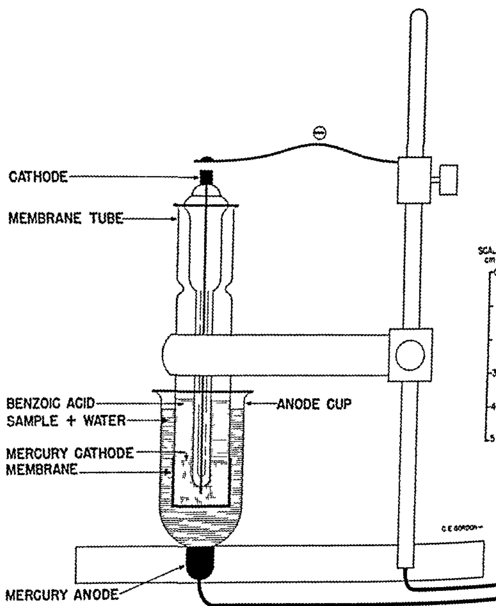


Fig 15

been used successfully two months later To remove the membranes from dried membrane tubes the collodion seal should be soaked at least 15 minutes in water It then merely becom s necessary to run the fingernail down the collodion seal and remove the old membrane in one piece

6 Krogh syringe pipettes 0 2 ml capacity and 1 7 ml capacity

Reagents

1 Water glass distilled over alkaline permanganate It must meet the specifications for conductivity water

2 Mercury base free Prepare this by clean ng w th 5% nitric acid with subsequent thorough washing with distilled water

3 Stock solution of benzoic acid (C H COOH) 1 000 N Dry benzoic acid by placing it in a vacuum desiccator at least 48 hours Accurately weigh 122 047 gm dissolve in 500 ml absolute EtOH and make up to liter with absolute EtOH. If stored n the cold it can be kept indefinitely

4 Benzo c ac d 0 0 N made from stock solution Pipette exactly 5 ml of 1 000 N benzoic acid nto a 50 ml volumetric flask Dilute to about 00 ml with glass distilled water with persistent agitation reprecipitated benzoic acid will di solve Dilute to mark

5 Sodium hydroxide 0 200 N (Kahlbaum CO free)

6 Phenolphthalein 0 10% (Lamotte) dilute 1 1 with EtOH

7 Collodion (Mallinckrodt) C P

8 Standard solution of mixed ions Dissolve and dilute to exactly one liter with glass dist lled water 8 190 gm NaCl 0 73 gm KCl 0 018 CaCl H O and 0 095 gm MgCl The result ng solution contains per liter 140 mEq Na 5 mEq K 5 mEq Ca^{++} and mEq Mg^{+} making a total base of 152 mEq per liter

9 Concentrated nitr c a id

Procedure

1 Wash anode cups with soap o Dreft rinse and leave filled with concentrated HNO

Rinse anode cups with distilled water several times then once w th glass d stilled water

3 From a burette add to each cup approximately 0 5 ml mercury and with a broken tip p pette approximately 10 ml of glass d stilled wate

4 Add 0 2 ml serum cells or urine or weighed tissue approximating 0 gm (the quantity u ed depends upon the base content) Rinse several times with water n anode cup keep ng the t p of the Krogh (automat c) pipette below the surface of the water to prevent splashing Then flush out pipette with 2 ml of water With each run have 0 2 ml of standard total base solution which has to be used for calculating the blank

5 Always rinse membrane tubes several times with distilled water and once with glass distilled water before use To each m mbrane tube add approx mately 1 ml of mercury

6 Add exactly a measured 1 7 ml of 0 02 N benzoic ac d The amount added de p nds on base n unknown (1 7 ml is equivalent to 170 mEq of base/liter of unknown when 0 2 ml of unknown is used)

7 Set membrane tubes in solution in anode cups submerged about 0 5 cm

8 Close circuit by attaching all electrode contacts and turn on current After letting it run low for 1 hour advance the rheostat and let it run ove n ght on h gh Dialys s is complete when m ll ammeter indicates no current or not more than 3 milliamperes for all 9 un ts

9 Remove contacts and lift membrane tube above solution in cups

10 Rinse off electrodes in membrane tubes with 2 ml glass distilled water

11 Add 1 drop of phenolphthalein

1 Titrate to first sign of pink color with 0.2000 N NaOH using Rehberg burette

Calculation

1 Blank

In this method the reagent blank is best computed from a standard solution not from a run of distilled water

mEq blank = (mEq acid − mEq alkali) − (ml standard × mEq total base per ml standard)

2 Unknown

mEq total base/liter unknown =

$$(\text{mEq acid} - \text{mEq alkali} - \text{mEq blank}) \times \frac{1000}{\text{ml unknown}}$$

Example

1 7 ml acid used titration of 0 2 ml of unknown is 0 0 1 ml NaOH titration of 0 2 ml of standard is 0 0034 ml NaOH

1 Blank = (1 7 × 02) − (0 0034 × 0 2) − (0 152 × 0 2) = 0 003

2 Unknown = (1 7 × 02) − (0 021 × 0 2) − (0 003) × $\frac{1000}{0\ 2}$ = 135 mEq/liter

3 This computation is made more expeditious by the use of Table 3

TABLE 3 TOTAL BASE

Milliequivalents Total Base per Liter of Serum Whole Blood Cells or Urine When 0 2 ml *Samples Are Analyzed When 1 7 ml of 0 02 N Benzoic Acid Is Used in the Cathode* Cups During Electrodialysis and When A Means Milliequivalents (NaOH Plus Blank) in Back Titration

A	0000	0001	000	0003	0004	0005	0006	0007	0008	0009
	MILLIEQUIVALENTS TOTAL BASE PER LITER UNKNOWN									
000	170	170	169	169	168	168	167	167	166	166
001	165	165	164	164	163	163	16	162	161	161
002	160	160	159	159	158	158	157	157	156	156
003	155	155	154	154	153	153	152	15	151	151
004	150	150	149	149	148	148	147	147	146	146
005	145	145	144	144	143	143	14	142	141	141
006	140	140	139	139	138	138	137	137	136	136
007	135	135	134	134	133	133	132	13	131	131
008	130	130	129	1 9	128	1 8	127	127	126	1 6
009	125	125	124	1 4	123	123	12	122	1 1	121
010	1 0	120	119	119	118	118	117	117	116	116
011	115	115	114	114	113	113	112	112	111	111
012	110	110	109	109	108	108	107	107	106	106
013	105	105	104	104	103	103	102	102	101	101
014	100	100	99	99	98	98	97	97	96	96
015	95	95	94	94	93	93	9	9	91	91
016	90	90	89	89	88	88	87	87	86	86
017	85	85	84	84	83	83	82	82	81	81
018	80	80	79	79	78	78	78	78	77	77
019	5	75	74	74	73	73	72	7	71	71
020	70	70	69	69	68	68	67	67	66	66
021	65	65	64	64	63	63	6	62	61	61
022	60	60	59	59	58	58	57	57	56	56
023	55	55	54	54	53	53	52	52	51	51
0 4	50	50	49	49	48	48	47	47	46	46
0 5	45	45	44	44	43	43	42	4	41	41
026	40	40	39	39	38	38	37	37	36	36
027	35	35	34	34	33	33	32	32	31	31
0 8	0	30	29	9	28	8	27	27	26	6
029	25	25	24	4	3	23	2	2	21	1
030	20	20	19	19	18	18	17	17	16	16
031	15	15	14	14	13	13	12	1	11	11
032	10	10	9	9	8	8	7	7	6	6
033	5	5	4	4	3	3	3	2	1	1
034	0	—	—	—	—	—	—	—	—	—

Precautions

1 In order to avoid erratic results only glass distilled water should be used.
2 Water gives irregular blanks therefore use one standard during each group of analyses to obtain the blank correction.

3 Sodium in Serum, Urine Cells Tissues Diets and Stools (Gravimetric Method)

A. IN SERUM

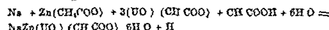
References

Butler A. M and Tuthill E An Application of the Zinc Uranyl Acetate Method for Determination of Sodium in Biological Material J Biol Chem 93 171 180 (Sept) *1931 193*

Consolazio W V and Dill D B The Determination of Sodium J Biol Chem 137 587 59 (Feb) 1941

Principle

The ash from biological material is treated with uranyl zinc acetate and the triple *salt of sodium uranyl zinc acetate is precipitated quantitatively* The precipitate is estimated g avimetrically



$Na + Zn(CH_3COO) + 3(UO)(CH COO) + CH COOH + 6H O \rightleftharpoons$

$NaZn(UO)(CH COO) 6H O + H$

Apparatus

1 *Jena* or Pyrex sintered glass filters (fine)
2 Platinum or quartz crucibles 0 ml capacity
3 Small electric stirring motor with platinum wire
4 Muffle oven set at 600 C
5 Electric centrifuge or good analytical filter paper (Whatman #40) and funnels
6 Syringe pipettes 1 0 ml and 0 ml
7 Analytical balance
8 An oven set at 110 C
9 Rubber stoppers #6 solid
10 Watch glasses
11 Source of su tion

Reagents

1 Uranyl zinc acetate (UZA)
   a. 80 gm of sodium free uranyl acetate $UO(C_2H O) 2H_2O$
      46 ml of acetic acid (CH COOH) 30% by volume
      Water to make up to 500 gm
   b. 0 gm of zinc acetate Zn(CH COO) 2H O
      23 ml of acetic acid 30% by volume
      Water to make up to 5 0 gm

Warm both solutions on steam bath Mix while hot let stand 24 hours and seed with sodium uranyl zinc acetate Stir well and filter before using

2 Ethyl alcohol (EtOH) 95% saturated with sodium uranyl zinc acetate Filter before use
3 Sulfuric acid concentrated
4 Ethyl ether anhydrous $(C_2H)O$
5 Calcium hydroxide powdered (sodium free)
6 Hydrochloric acid one N (10 ml concentrated HCl in 100 ml of water)
7 Sod um uranyl z nc acetate (triple sodium salt)

Procedure

1. Pipette exactly 1 ml of serum into a platinum or quartz crucible
2. Add one drop of concentrated sulfuric acid and char at 110 C for at least 18 hours.
3. Ash in muffle oven (600 C) overnight
4. To the bottom of an *accurately weighed sintered glass filter fit a* #6 rubber stopper
5. Add approximately 15 ml of freshly filtered UZA.
6. To the ashed serum add 5 to 7 drops of one N hydrochloric acid.
7. By means of a medicine dropper transfer the sample quantitatively to the weighed crucible while mechanically stirring the UZA
8. Wash remaining serum ash with three 0 2 ml portions of approximately 0 1 N HCl *transferring it to the crucible*
9. Now wash the crucible with 2 ml of UZA.
10. Stir mechanically for a total of 5 minutes Then *rinse the stirring rod with 2* ml of UZA cover the crucible with a watch glass and let stand *for* 30 *minutes* at room temperature
11. Filter the ingredients in crucible by suction
12. Using capillary pipettes, wash 5 times with 2 ml portions of freshly filtered 95% alcohol saturated with the triple salt of UZA.
13. Then wash 5 times with 2 ml portions of absolute ether
14. Dry by vacuum Clean outside of crucible with a *clean cloth and place in a desiccator* for 1 hour
15. Weigh

Calculation

1. If 1 0 ml of serum was used mEq Na/liter = (weight of precipitate in grams − blank in grams) × 650
2. *To convert mEq Na to mg Na, multiply by 23*

Example

1. Weight of precipitate was 0 2220 gm and weight of blank was 0 00?0 gm, mEq Na/liter serum = (0 2 .0 − 0 0020) × 6.0 = 143 0 mEq/l
2. mg Na = 143 0 × .3
3. This calculation is assisted by Table 4.

Precautions

1. *In washing out the precipitate be sure that no water is added as the* sodium zinc uranyl acetate precipitate dissolves very readily

TABLE 4 SERUM SODIUM

When 1 ml of serum is analyzed, mEq Na/liter =
(gm ppt − gm blank) × 650 or
(mg ppt − mg blank) × 0 65

MG PPT MINUS MG BLANK	0	1	2	3	4	5	6	7	8	9
	MILLIEQUIVALENTS SODIUM PER LITER SERUM									
190	1 4	124	1 5	1.6	1 6	1 7	1.7	1 8	1.9	1 9
200	1.0	131	131	132	133	133	134	1 5	135	136
210	137	137	138	1.8	139	140	140	141	14.	14?
220	143	144	144	145	146	146	14.	148	149	149
230	150	150	1.1	151	15?	153	153	154	15	155
240	1.6	1.7	157	1.8	1.9	159	160	161	161	16
250	163	163	164	164	16.	166	166	16	168	168

2 Make sure that the filters are dry before weighing
3 The accuracy of the method must be checked against a standard solution (see total base)

B SODIUM IN URINE

Apparatus See serum
Reagents See serum

Procedure

1 To approximately 10 ml of urine add approximately 1 gm of powdered $Ca(OH)$
2 Shake manually at intervals for 1 hour
3 Centrifuge or filter through hardened filter paper

TABLE 5 SODIUM IN URINE

When 2 ml of urine are used for analysis mEq Na/liter urine =
(gm ppt − gm blank) × 325 or
(mg ppt − mg blank) × 0 3 5

MG PPT MINUS MG BLANK	0	1	2	3	4	5	6	7	8	9
	MILLIEQUIVALENTS SODIUM PER LITER URINE									
0	0 0	0 3	0 7	1 0	1 3	1 6	0	3	2 6	9
10	3 3	3 6	4 0	4 2	4 6	4 9	5 3	5 6	9	6
20	6 5	6 8	7 2	7.5	7 8	8 1	8 5	8 8	9 1	9 4
30	9 8	10 1	10 4	10 7	11 1	11 4	11 7	1 0	1 4	12 7
40	13 0	13 3	13 7	14 0	14 3	14 6	1 0	1 3	1 6	15 9
50	16 3	16 6	16	17	17 6	17	18	18 5	18 9	1 2
60	19 5	19 8	0 2	0 5	0 8	21 1	1 5	21 8	2 1	4
70	2 8	23 1	3 4	3 7	4 1	4 4	24 7	2 0	4	2 7
80	26 0	26 3	26 7	27 0	27 3	7 6	28 0	8 3	28 6	8 9
90	9 3	9 6	9 9	30 2	30 6	0 9	31 2	31 5	31 9	[illegible]
100	3 5	32 8	33	33 5	33 8	34 1	34 5	34 8	3 1	35 4
110	35 8	36 1	36 4	36 7	37 1	37 4	37 7	38 0	38 4	38 7
1 0	39 0	9 3	9 7	40 0	40 3	40 6	41 0	41 3	41 6	41 9
130	4 3	42 6	4 9	43 2	43 6	43 9	44 2	44 5	44 9	45
140	45 5	45 8	46	46 5	46 8	47 1	47 5	47 8	48 1	48 4
150	48 8	49 1	49 4	49 7	50 1	50 4	50 7	51 0	51 4	51 7
160	5 0	2 3	5 7	53 0	53 3	53 6	54 0	4 3	54 6	54 9
170	53	55 6	55 9	56	56 6	56 9	57 2	57 5	5 9	58 2
180	58 5	58 8	59	9 5	59 8	60 1	60 5	60 8	61 1	61 4
190	61 8	6 1	6 4	6 7	63 1	63 4	63 7	64 0	64 4	64 7
200	6 0	6 3	6 7	66 0	66 3	66 6	67 0	67 3	67 6	67 9
10	68 3	68 6	68 9	69 6	69 6	69 6	70	70 5	70 9	71
0	71	71 8	7 2	7	7 8	73 1	73 5	3 8	4 1	4 4
230	74 8	75 1	5 4	75 7	76 1	76 4	6	77 0	7 4	77 7
40	78 0	78 3	78 7	9 0	79 3	79 6	80 0	80 3	80 6	80 9
250	81 3	81 6	81	8	8 6	8 9	83 2	83 5	83 9	84 2
260	84	84 8	85	85 5	85 8	86 1	86 5	86 8	87 1	8 4
70	87 8	88 1	88 4	88 7	89 1	89 4	89 7	90 0	90 4	90 7
280	91 1	91 3	91 7	9 0	9 3	9 6	93 0	9 3	93 6	93 9
290	94 3	94 6	94 9	95 2	95 6	59	96	96 5	9 9	97
300	97 5	97 8	98	98	98 8	99 1	99 5	8	100 1	100 4
310	100 8	101 1	101 4	101 7	102 1	10 4	10	103 0	103 4	103 7
3 0	104 0	104 3	104 7	105 0	105 3	105 6	106 0	106 3	106 6	106 9
330	107 3	107 6	107 9	108	108 6	108 9	109 2	109 5	109 9	110
340	110 5	110 8	111	111 5	111 8	112 1	11 5	11 8	113 1	113 4
350	113 8	114 1	114 4	114 7	115 1	115 4	115 7	116 0	116 4	116 7
360	117 0	117 3	117 7	118 0	118 3	118 6	119 0	119 3	119 6	119 9
370	1 0 3	120 6	120 9	1 1	1 1 6	1 1 9	1	1 5	1 9	1 3
380	1 3 5	1 3 8	1 4	124 5	1 4 8	1 5 1	1 5 5	1 5 8	126 1	1 6 4
390	126 8	1 7 1	1 7 4	127 7	128 1	1 8 4	128 7	1 9 0	129 4	129 7

5 To the ash add 0 ? ml of 20% H_2PtCl_6
6 Agitate the tube *for ½ to one minute or until complete solution occurs and* K_2PtCl_6 begins to precipitate
7 Add approximately 5 ml absolute EtOH and mix contents thoroughly with stirring rod Rinse the rod with 1 2 ml absolute EtOH.
8 Let the tubes stand for 30 minutes
9 Centrifuge gently for 10 minutes at about 1500 r p m
10 Aspirate or decant supernatant liquid. (Save it for platinum recovery if desired)
11 Wash contents with 5 ml of absolute EtOH mix with stirring rod and rinse rod as before
12 Centrifuge again for 2 3 minutes at 1500 r p m, aspirate the supernatant liquid and save residues
13 Place tubes in 70 C water bath
14 Bring temperature to boiling and slowly maintain for 5 10 minutes until EtOH is completely volatilized If EtOH is not completely evaporated it will reduce the iodoplatinate to free platinum black
15 Leave the tubes in hot water and add approximately 4 5 ml of water to each.
16 *Dissolve salt* (K_2PtCl_6) *completely If the salt is not dissolved free platinum* will be formed upon addition of KI.
17 Add approximately 1 ml 2 N KI mix and let stand 5 minutes in hot water
18 Then cool and titrate with 0 2 ml Rehberg burette using 0 1000 N $Na_2S_2O_3$ until complete disappearance of red color The remaining solution will be lemon yellow Save the residue for recovery of the platinum present

Calculation

If 1 ml serum used mEq K /liter serum = ml of $Na_2S_2O_3$ × normality × 1000

To convert mEq potassium per liter to mg potassium per 100 ml multiply mEq/liter by 3 9 (i.e equivalent weight of potassium × 1/10)

Example

Titration figure was 0 0530 ml of 0 1000 N sodium thiosulfate and the blank was 0 0030 ml

mEq K /liter of serum = (0 0530 − 0 0030) × 0 1000 × 1000 = 5 0

Precautions

1 Make certain that all the alcohol is evaporated before adding the water
2 Then make certain that the precipitate is dissolved in the water before adding the potassium iodide (If not free platinum will be formed upon addition of the KI.)
3 Be sure that the ash is white otherwise muffle again
4 It is wise to check the accuracy of the method with a standard solution (see total base)

B POTASSIUM IN RED BLOOD CELLS

Reference

Consolazio W V (Unpublished)

1 Pipette exactly 0 1 ml of cells with automatic syringe pipette into quartz or Vycor tube as for serum
2 Add 1 drop of saturated sodium sulfate (Na_2SO_4)
3 Add 1 drop of H_2SO_4 Dry at 110 C
4 In order to ash add 1 drop of concentrated HCl and place in 100 C oven until dry This step prevents occlusion of K salts on the iron, since all ferric oxide is converted to ferric chloride
5 Now proceed as for Serum, steps 4 18

C POTASSIUM IN URINE

1 Into a quartz or Vycor tube p pette 0 2 ml of urine
Add 1 drop of sodium sulfate solution
3 Add 1 drop of sulfuric acid solution
4 Proceed as for Serum steps 3 18

D POTASSIUM IN DIET STOOLS AND TISSUE

1 Use approximately 0 5 to 1 gm for food feces or tissue Ash in muffle
To the proper amount of HCl extract of ash add 1 drop of Na_2SO Do not add $H SO_4$.
Dry at 110 C Do not ash
4 Proceed as for Serum steps 5 18
5 Pyrex tubes may be used here in place of quartz tubes since no ashing is employed after step

5 Calcium in Serum Urine Food Tissue and Feces

A. IN SERUM

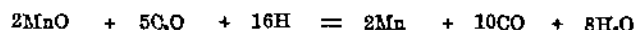
References

Kramer B and Tisdall F F A S mple Technique for the Determination of Calcium and Magnesium in Small Amounts of Serum J Biol Ch m 47 475 481 (Aug) 19?1

Clark E P and Collip J B A Study of the Tisdall Method for the Determination of Blood Serum Calcium With a Suggested Modification J Biol Chem 63 461 464 (March) 19?5

Principle

Calcium is precipitated as the oxalate which is titrated in hot acid solution against standard potassium permanganate



$$2MnO + 5C_2O + 16H = 2Mn + 10CO + 8H_2O$$

Apparatus

1 A quantity of 15 ml conical centrifuge tubes
A water bath with boiling water
3 A 3 or 5 ml microburette
4 An electric centrifuge
5 Accurately cal brated 1 and 2 ml syringe pipettes

Reagents

1 Phenol red approximately 0 04% in water
2 Ammonium oxalate approximately 45 gm per liter
3 Ammonium hydroxide approximately 0 35 N i.e approximately 2 ml of concentrated NH_4OH diluted to 100 ml with water
4 Sulfuric ac d approx mately N prepared by diluting 3 ml concentrated $H SO$ to 100 ml with water
5 Standard potass um permanganate 0 01 N This is best prepared from a well aged 0 1 N standard (3 161 gm $KMnO$ per l) and should be standardized weekly aga nst oxalate as follows
   a A standard 0 1 N solution of sodium oxalate is prepared by diluting exactly 6 700 gm of pure dry Na_2C_2O to exactly one liter
   b A standard 0 01 N solut on is prepared by diluting exactly 10 ml of the 0 1 N solution to exactly 100 ml.

c In conical tubes place exactly 2 ml of the 0 01 N standard and approximately 2 ml of 1 N H SO Heat in the water bath at 60 70 C and *titrate while hot* against the dilute KMnO

d Calculation Normality of dilute $KMnO_4 = \frac{2}{\text{Titration figure in ml}} \times 0\ 01$

6 Ammonia Approximately a 2% solution

7 Calcium Standard (Primary Standard)
0 250 gm of dry calcium carbonate (pure Iceland spar) in a small amount of wat in a 1000 ml flask Add some dilute HCl (1 10) to dissolve the crytals and the add a few ml of acid in excess Dilute to 1000 ml with water and mix This sol tion is stable indefinitely and contains 0 1 mg of Ca per ml It corresponds to serum with a Ca content of 10 mg/100 ml

Procedure

1 *Pipette exactly 2 ml of serum into a conical tube*

2 Add approximately 1 ml of ammonium oxalate and 1 drop of phenol red. Mix b rotation of the tube

3 *Add 2% ammonia drop by drop until the indicator turns red*

4 Stand at room temperature for at least .0 minutes for precipitation and then ce trifuge at high speed for 10 minutes Do not use an angle head centrifuge

5 *Using a fine capillary and slow suction, suck off and discard the supernatant flu* taking great care not to lose any of the precipitate Do not try to decant th supernatant

6 Pipette approximately 3 ml of 2% NH OH down the sides of the tube and st the precipitate by twirling the tube by hand.

7 Centrifuge at high speed for 3 minutes suck off the supernatant fluid and repea steps 6 and 7 until no color of phenol red remains in the precipitate *Wash 4 time in this way*

8 Tap the tube sharply to loosen the precipitate and add 2 ml of 1 N H_2SO

9 Place tubes in boiling water until the precipitate dissolves

10 Titrate with 0 01 N KMnO to the first permanent pink color lasting 30 seconds Tube and contents must never become cooler than 70 C during the titration.

11 With each run set a reagent blank consisting of 2 ml of 1 N H SO and titrat as in Steps 9 and 10 The blank should not be above 0 05 ml if reagents are sat isfactory

Calculation

mg Ca/100 ml serum =

$\frac{(\text{ml titration} - \text{ml blank})}{2} \times$ Normality KMnO $\times$ .0 04 $\times$ 100 =

(ml titration − ml blank) $\times$ Normality KMnO $\times$ 1002

Example

Normality of KMnO was 0 0095 Titration was 1 10 ml Blank was 0 03 ml.
Therefore mg Ca per 100 ml serum = (1 10 − 0 03) (0095) (1002) = 10 2

Precautions

1 The phenol red turns reddish purple in Step 3 never true purple

2 Take great care not to lose precipitate in Steps 5 6 and 7

3 The titration must be carried out above 70 C When the first drop of KMnO is added it persists for some time As soon as some *manganous salt is formed*, the titration proceeds more rapidly to the end point

4 *Wash at least 4 times with ammonia* The amount and type of washing should be determined for each laboratory It depends upon shape of tubes efficiency of centrifuge and probably the quality of reagents especially distilled water In our laboratory we get very erratic results with only two washings
5 Be sure that the distilled water is of constant quality free of calcium

B CALCIUM IN URINE

Reference

(Same as for serum)

Principle

Same as in serum calcium The presence in urine of magnesium iron and phosphate in significant amounts makes attention to the acidity during precipitation very important

Apparatus

(Same as for serum)

Reagents

1 Phenol red sulfuric acid and standard potassium permanganate as in serum calcium
2 Ammonium hydroxide 4N Dilute ? ml of concentrated NH_4OH to 100 ml with water
3 Acetic acid Dilute 10 ml of glacial acetic acid to 100 ml with water
4 Sodium acetate saturated Approximately 100 gm are added to 100 ml of water
5 Oxalic acid 1 N Dissolve 63 gm of acid in one l of water

Procedure

1 Use freshly collected urine without added stabilizers particularly *without added oxalate* Pipette exactly 1 ml of urine into a conical tube
2 Add 1 drop of phenol red and then 4 N NH_4OH drop by drop until the indicator is pink.
3 Quickly add 10% acetic acid drop by drop until indicator turns yellow
4 Add 1 ml of oxalic acid and 1 ml of sodium acetate
5 Mix well and stand 1½ hours at room temperature
6 Proceed exactly as in Steps 4 through 10 in serum calcium

Calculation

mg Ca per 100 ml urine =

$$\frac{(\text{ml titration} - \text{ml blank})}{\text{ml urine}} \times (\text{normality } KMnO_4) \times 20\,04 \times 100 =$$

(ml titration − ml blank) × normality $KMnO_4$ × 2004

Example

Normality of $KMnO_4$ was 0 0096 Titration was 1 55 ml. Blank was 0 05 ml
Therefore mg Ca per 100 ml urine = (1 55 − 0 05) (0 0096) (2004) = 28 8

Precautions

1 Use fresh urine before precipitation has taken place If the urine must be allowed to stand add 4 ml of glacial acetic acid per 100 ml of urine and correct appropriately in the calculations
2 Other precautions as in serum calcium
3. Wash at least 4 times with ammonia. The exact number of washings has to be determined for each laboratory

4 This method is not very specific Under some conditions as during therapy with sulfaguanidine, urine contains substances which interfere with the estimation Under such conditions the urine has to be ashed with sulfuric acid 3 drops of concentrated sulfuric acid to every 2 ml of urine

C CALCIUM IN FOOD TISSUE AND FECES

Reference

Corley, R C, and Denis W The Determination of Calcium in Tissue Feces and Milk J Biol Chem 66 601 608 (Dec) 1925

Apparatus

1 A quantity of porcelain or platinum crucibles
2 Muffle oven set at 600 C
3 A quantity of good analytical filter paper (Whatman #40) and funnels
4 Rubber stoppers
5 Rest of apparatus same as for serum

Reagents

1 Concentrated nitric acid.
2 Hydrochloric acid one ml concentrated HCl to two parts of water
3 Alizarin red indicator in water
4 Litmus paper
5 Oxalic acid, 2 5 percent in water
6 Ammonium oxalate 3 percent in water
7 Ammonium oxalate, 0 5 percent in water
8 Sodium acetate 20 percent in water
9 Sulfuric acid (1 N)
10 Concentrated ammonia
11 The rest of the reagents are the same as for serum

Procedure

1 For food tissue and feces use samples weighing approximately 2 gm
2 Ignite in a porcelain or platinum crucible to a white ash with the aid of a few drops of conc HNO_3 and dissolve with HCl
3 Bring the volume to approximately 75 to 150 ml.
4 Make alkaline with concentrated ammonia adding it drop by drop Use litmus or alizarin red as an indicator
5 Then add concentrated HCl drop by drop until just acid to litmus
6 Add 10 drops of concentrated HCl (sp gr 1 20)
7 Add 10 ml of 2 5% oxalic acid
8 Stopper flask with a rubber stopper mix and stand for 12 hours
9 Add an excess of 3% ammonium oxalate and cool to room temperature
10 Then add 8 ml of 20% sodium acetate solution (For feces use 15 ml)
11 Stopper and shake vigorously for 10 minutes
12 Filter and wash precipitate with 0 5% ammonium oxalate (This is to wash free of chloride)
13 Then wash 3 times with cold distilled water The exact number of washings has to be determined in each laboratory
14 Transfer precipitate quantitatively to a 50 ml beaker washing last traces off filter paper with approximately 5 ml of 1 N sulfuric acid
15 Add approximately 25 ml of water and stir until precipitate is all dissolved. It may be necessary to add more sulfuric acid or to warm gently
16 Dilute with water to exactly 100 ml in a volumetric flask.
17 Titrate 10 ml aliquots with 0 01 N $KMnO_4$ as for urine

Calculation

mg Ca per 100 gm sample =

$$\frac{(\text{ml titration} - \text{ml blank})}{(\text{gm sample} \times 10/100)} \times (\text{normality } KMnO_4) \times (20.04) \times (100)$$

Example

A sample of food weighed 1 9546 gm Normality of $KMnO_4$ was 0 0095
Sample was diluted to 100 ml and 10 ml aliquots were titrated Titration figure was 50 ml Blank was 0 04 ml

mg Ca/100 gm food =

$$\frac{(2\ 50 - 0\ 04)}{(1\ 9546 \times 10/100)} \times 0\ 0095 \times 20\ 04 \times 100 = 239\ 5$$

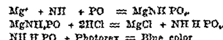
6 Magnesium in Serum

Reference

Briggs A P A Colorimetric Method for the Determination of Small Amounts of Magnesium J Biol Chem 52 349 355 (June) 192

Principle

Magnesium is precipitated as magnesium ammonium phosphate and the phosphate is estimated by the colorimetric method of Tschopp and Tschopp or that of Fiske and Subbarow



$Mg^{++} + NH_4^+ + PO_4^{---} = MgNH_4PO_4$
$MgNH_4PO_4 + 2HCl = MgCl_2 + NH_4H_2PO_4$
$NH_4H_2PO_4$ + Photorex = Blue color

Apparatus

1 A quantity of 15 ml conical centrifuge tubes
2 An electric centrifuge
3 A quantity of rubber policemen
4 Refrigerator
5 Water bath set between 60 70 C
6 Coleman Jr Spectrophotometer Model 6
7 Cuvettes 19 × 150 mm

Reagents

1 Monobasic potassium phosphate (KH_2PO_4) approximately 4% solution in water
2 Ammoniacal alcohol This is prepared from 200 ml of 95% EtOH and 50 ml of concentrated NH_4OH diluted to one l with water
3 Trichloracetic acid (CCl_3COOH) 15% solution in water Store in refrigerator
4 Tschopp and Tschopp phosphorus reagents (See phosphorus method for reagents)
5 Ammonium oxalate ($(NH_4)_2C_2O_4 \cdot H_2O$) approximately 40% in water
6 Concentrated ammonium hydroxide (NH_4OH) low in magnesium and phosphate
7 Hydrochloric acid approximately 1 N and N/3
8 Standard magnesium solution 0 1413 gm of magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6H_2O$) per liter in approximately 0 01 N sulfuric acid Add 2 ml of chloroform for a preservative Of this 5 ml = 0 07 mg Mg
9 Molybdic acid photorex and sodium bisulfite as described in the method on phosphorus

Procedure

1 Mix in a 15 ml conical centrifuge tube

2 0 ml of serum
2 0 ml of water
1 0 ml of ammonium oxalate solution

Mix by inversion and centrifuge
(The precipitate may be used for determination of calcium)

2 Into a conical centrifuge tube pipette 4 0 ml of the supernatant liquid
3 Add 6 0 ml of 15% trichloracetic acid, mix and centrifuge
4 Transfer 5 0 ml of supernatant fluid to cone tipped 15 ml centrifuge tube
5 Add 1 ml of 2% potassium acid phosphate solution (KH_2PO_4)
6 Add 4 ml of concentrated ammonia (NH_4OH)
7 Scratch side of tube with rubber policeman until precipitate forms and rinse rod with a few ml of water
8 Let stand overnight in refrigerator
9 Centrifuge decant and wash with 5 ml of ammoniacal alcohol
10 Centrifuge and decant again
11 Dissolve the precipitate in 1 ml of 1 N HCl
12 Transfer quantitatively to a calibrated 25 ml colorimeter tube
13 Estimate phosphate (equivalent to magnesium) by adding the following phosphate reagents

0 4 ml of $NaHSO_3$
0 4 ml of molybdic acid
0 4 ml of Photorex

14. Dilute to exactly 25 ml
15 Heat for 20 minutes at 60 70 C in water bath then cool under running water
16 Set reagent blank at 100% T at wave length 660 mm and read unknowns

Calculation

1 A standard curve is based on samples containing 0 01 to 0 05 mg of magnesium per milliliter and treated as above
2 mg of magnesium per 100 milliliters of serum =

$$(\text{mg magnesium in cuvette}) \times \frac{2}{(5)} \times \frac{4}{(10)} \times 100 =$$

(mg magnesium in cuvette) × 16

Example

Magnesium in cuvette was determined to be 0 04 mg
mg magnesium/100 ml serum = 0 04 × 16 = 2 4

Precautions

1 All reagents must be as low in magnesium as it is possible to procure them

7 Inorganic Iron in Serum

Reference

Kitzes G Elvehjem C A and Schuette H A The Determination of Blood Plasma Iron J Biol. Chem 155 653 660 (Oct) 1944.

Principle

Serum or plasma is boiled in water to form a gelatinous solution the proteins are precipitated out with trichloracetic acid and the filtrate is treated with alpha, alpha bipyridine to produce a pink color which is measured photo colorimetrically

Apparatus

1 Coleman Jr Spectrophotometer Model 6
2 Cuvettes 19 × 150 mm
3 A number of 15 ml con cal graduated centrifuge tubes
4 A number of 1 3 4 and 5 ml volumetric pipettes
5 A quantity of glass stirring rods
6 A constant temperature bath set at 90 95 C
7 An electric centrifuge

Reagents

1 Trichloracetic acid 5% in water If not free of iron this acid should be re d stilled.
Ind cator p nitrophenol 0 1% solution in water
3 Ammon um hydroxide solution approximately 6 N in water
4 Buffer solut on (pH 4 58) To prepare this 27 2 ml of glacial acetic acid and 33 4 gm of anhydrous sodium acetate are dissolved in water and made up to 250 ml
5 Thioglycolic ac d solut on (Eastman Practical Grade)
6 Alpha alpha bipyrid ne reagent Dissolve 0 gm of reagent in 5 ml of glacial acetic acid and dilute to 100 ml with water
7 Redistilled water (iron free) Redistill in a glass still
8 Standard iron solution D ssolve iron wire (99 8%) in a mixture of n tric acid and hydrochloric ac d and dilute with water to give a concentration of 1 mg/ml From this solut on standard solutions of 100 and 1 mcg per ml are made

Procedure

1 Pipette exactly 3 to 5 ml of plasma or serum (containing approximately 3 to 9 mcg of inorgan c iron) into a 15 ml conical centrifuge tube
2 Add 3 ml of red stilled water and mix with plasma
3 A blank should be prepared at the same time using all the reagents
4 Place the tubes in boiling water for ° 3 minutes or until the solution becomes opaque This must be done very carefully so as not to get a gelat nous coagulation from which it will be difficult to extract the iron
5 Remove the tubes wh le the solution is still liquid and opaque and allow to cool by plac ng them n a beaker of cold water
6 Add ml of trichloracetic acid (5%) stir carefully and thoroughly so that the acid is mixed intimately with the solution The precipitate that is formed becomes fine and flocculant A small blunt end stirring rod is preferred so as not to run the end of it through the centrifuge tube
7 Place the tubes in a water bath set between 90 95 C for about three minutes stirring the solution once or twice
8 Remove the tubes from the bath and cool again in cold water
9 Then centrifuge the tubes at 2000 to 3000 r p m for about five minutes
10 Remove the tubes and carefully decant the supernatant fluid into a 15 ml graduated tube
11 To the precipitate remaining in the tubes add 4 ml of redistilled water and I ml trichloracetic ac d Then break up the precipitate and stir it well
1 Aga n place in the water bath (90 95 C) for 3 minutes stirring each solution once or twice Remove and cool the tubes in cold water
13 Centrifuge the tubes for 5 minutes and then decant the supernatant fluid into the tube containing the first filtrate
14 Add 1 drop of p nitrophenol ind cator and then add ammonium hydroxide solut on drop by drop until the solution becomes yellow
15 Add 1 ml of the buffer solution and dilute to exactly 15 ml. Mix thoroughly and cent ifuge

16 Pipette 10 ml into a cuvette and then add 2 drops of thioglycolic solution. *(Do not get this solution on fingers or skin as it is very toxic.)* Mix by tapping the tube or by a slight rotary motion

17 Add 1 ml alpha alpha'bipyridine reagent and a faint pink color will develop Mix carefully but thoroughly

18 Set the blank at 100% T at 510 mμ and read the unknowns and blank

Calculations

A standardization curve is set up on the Coleman Jr Spectrophotometer with standard solutions of iron ranging from one gamma to twenty gamma The calibration constant K is computed as in the section on spectrophotometry

Then mcg inorganic iron/100 ml serum =

$$\frac{K(2 - \log \% T) \times 15 \times 100}{\text{ml aliquot} \times \text{ml serum}}$$

Example

Five ml of serum were analyzed K was 45 32 %T'' was 89 5 Aliquots of 10 ml were used.

mcg inorganic iron/100 ml serum =

$$\frac{45\ 32\ (2 - \log 89\ 5) \times 15 \times 100}{10 \times 5} = 113\ 5$$

Precautions

Thioglycolic acid is very toxic Be careful with it

8 Copper in Serum

A. METHOD I

Reference

Robinson J C A Simple Method for the Determination of Serum Copper J Biol Chem 179 1103 1109 (July) 1949

Principle

The addition of sodium diethyldithiocarbamate to a solution containing copper produces a golden brown color that can be measured colorimetrically The copper salt can be rapidly and quantitatively extracted from an aqueous solution by isoamyl alcohol which also intensifies the color This color is stable for at least two hours at a pH between 5 7 and 9 2

Iron interferes with the color but the addition of sodium pyrophosphate converts it to iron pyrophosphate which does not react with the carbamate

Apparatus

1 Test tubes 13 × 100 mm with ground glass stoppers
2 Pipettes. Serological 0 2 ml graduated to 0 01 ml
3 Mechanical shaker Kahn
4 A water bath set between 50 to 60 C
5 Spectrophotometer In this laboratory a Coleman Model 6 equipped with 1 × 75 mm cuvettes is used
6 Syringe pipettes exactly 0 1 ml 0 2 ml 1 0 ml and 3 0 ml.
7 Glass still for metal free water
8 Electric centrifuge

Reagents

1 Sod um pyrophosphate Satu ate red st lled water w th the salt

° Ammon um hydroxide 10 to 12% D st ll concentrated ammon a (°8% reagent) nto red st lled water to saturat on D lute to requ red concentrat on w th red s t lled water

3 Sod um d ethyld th ocarbamate °% aqueous solut on Remove copper by shaking w th carbon tetrachlor de wh ch has been dist lled over calc um ox de The solu t on s stable for some weeks f stored n the dark. (Sod um diethyld th o ca bamate the reagent used to react w th copper n th s procedu e gives colored complexes with a number of heavy metal cat ons In b olog cal material how ever on s the ch ef nterfer ng substance Th s nterference of iron s sat s fa tor ly el m nated by develop ng the copper carbamate complex n an am mon acal solut on conta ning pyrophosphate and hav ng pH 9 or greater The color ntens t es of other nterfering metals such as n ckel and cobalt are we ght for we ght only 1/20 1/30 as g eat as that g ven by copper Because the con centrat on of these ons n serum s of such relat vely low order the r nterference s negl g b e)

4 Metal f ee water D st l ed water red stilled from res stant glass apparatus should be used n the preparat on of all reagents and n the final r ns ng of equ p ment

5 S anda d copper solut on 0 100 mg Cu/ml Prepa e stock solut on by d ssolving 0 9 8 gm of uneffio esced $CuSO$ $5H$ O n red st lled water and di ut ng to one l ter F om th s stock solut on the work ng standa ds may be prepa ed It s best to use a solut on conta n ng 1 mcg per ml because the final volume of the aqueous phase should be kept at a m n mum To p epa e th s the d lut on s 1 ml of stock solut on to 100 ml w th water

6 Isoamyl alcohol D st ll from all glass apparatus saturate w th water and store at 4 C

Procedure

1 Into ea h of g ass stoppered test tubes accu ately del ver 1 0 ml serum
Add to each of the tubes 0 2 ml of saturated solut on of sod um pyrophosphate Mix by tapp ng gently

3 To ea h of the tubes add exactly 0 04 ml of 10 1 % ammon um hyd ox de Aga n mix by tapp ng

4 Add 0 2 ml sod um d ethyld th oca bamate to one of the tubes and 0 ml water to the other wh ch s the serum blank M x and a low to stand for one hour

5 P pette exactly 3 0 ml of soamyl alcohol nto each tube

6 Place tubes n l ahn rack and secu ely fasten nto a horizontal pos t on on a mechan cal shaker (It s con en ent to p ace a sponge against one end of the shaker and clamp the rack so that the stoppered ends of the tubes are firmly presse l aga nst the sponge) Shake for 15 m nutes

7 Place tubes n cent fuge cups and cool unt l water used n balanc ng the cups beg ns to form crystals of ce

8 Centrifuge at 4000 r p m for 10 minutes If the m n mum requ red volume (1 5 ml for 1 × 75 mm cuvette) of extract s not obta ned gently shake the p ec p tate f ee nto the soamyl alcohol and repeat centr fugat on

9 Carefully p pette the soamyl alcohol fract ons nto 1° × 5 mm cuvettes stopper and measure opt cal dens t es at 440 mμ (If c oudy extracts are obta ned place cuvettes for a few seconds n water wa med to 50 60 C)

10 It s nece sary to p epare a reagent blank subst tut ng d st lled wate for serum for each series of determ nat ons Ve y low read ngs about 99 100 pe cent trans m tance are obta ned.

Calculations

Let O_c represent the optical density due to the serum copper, O the optical density of the carbamate extract from serum, O the optical density of the serum extract and O the optical density of the reagent blank

Then O_c = O − (O + O)

and Copper *mcg per 100 ml serum* =

O_c × calibration constant × dilution factor × 100

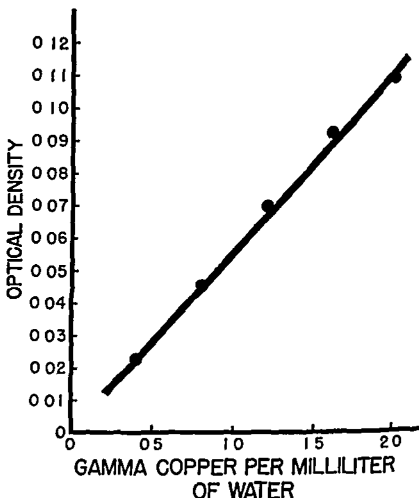


Fig 16 —Example of calibration curve for copper in serum Ordinate optical density abscissa copper mcg per ml of water

Example

O was 0 09 O was 0 06 O was 0 01, and K (mcg copper per ml per unit optical density) was 17.5

In the analysis, the copper of 1 ml serum was extracted into 3 ml of isoamyl alcohol therefore copper mcg per 100 ml serum =

$$(0\,09 - 0\,06 - 0\,01) \times 17\,5 \times \frac{3}{1} \times 100 = 105$$

Experimental Considerations

As prev ously mentioned the final color is due to three components serum copper copper as an impurity in the reagents and substances other than copper carbamate A large percentage of the density of the final color is due to materials extracted from the serum other than copper carbamate This point is demonstrated in Figs 16 and 17 which show that the max mum light absorption for both an i oamyl alco hol extract of copper carbamate from aqueous solution and an isoamyl alcohol ex tract of the serum blank occurs in the same general region of the spectrum

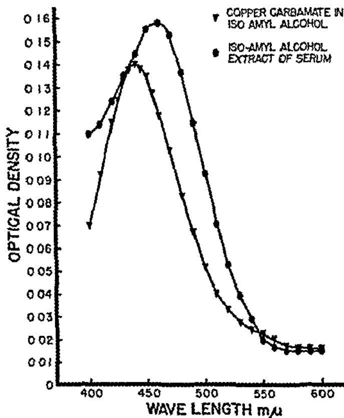


Fig 17—Spectral t an mis ion curv of copper arbamate and serum. Ordinate optical d n ity absci a wa e length in millimi ron

The ratio of color to the concentration of copper carbamate in isoamyl alcohol is linear up to a concentration of about 7 mcg Above this value however there is an appreciable de iation from Beer s law This should be borne in mind particu larly when doing recovery exper ments and in such experiments concentration *figures should be obtained from a calibrat on cur e rather than use of the cal bra t on constant*

Precautions

1 Be certain the tubes are cool before centrifug ng the specimens
2 All reagents must be nearly free of metals of the iron cobalt nickel ser es as well as of copper

B METHOD II

Reference

Cartwright E Jones J and Wintrobe M A Method for the Determination of Copper in Blood Serum J Biol Chem 160 593 600 (Oct) 1945

Principle

The addition of sodium diethyldithiocarbamate to a solution containing copper produces a golden brown color that can be measured colorimetrically The copper salt can be rapidly and quantitatively extracted from an aqueous solution by isoamyl alcohol which also intensifies the color This color is stable for at least _ hours at pH 5 7 to 9 2

Iron interferes with the color but the addition of sodium pyrophosphate converts it to iron pyrophosphate which does not react with the carbamate

Apparatus

1 Coleman Jr Spectrophotometer Model #6
2 Cuvettes 19 × 150 mm
3 A quantity of 15 ml centrifuge tubes graduated
4 Electric centrifuge
5 A water bath set at 90 95 C
6 A quantity of glass stirring rods
7 Glass water still

Reagents

1 Redistilled water is used in all the reagents
2 Trichloracetic acid 20% in water
3 Sodium pyrophosphate saturated solution
4 Ammonium hydroxide solution 10 to 1° percent
5 Sodium diethyldithiocarbamate solution 1%
6 Standard copper solution as used in the method of Robinson (1949)
7 Isoamyl alcohol

Procedure

All glassware used must be carefully cleaned and then rinsed three times with re distilled copper free water

1 Pipette 3 to 5 ml samples preferably 5 ml of serum or plasma into ungraduated 15 ml Pyrex centrifuge tubes It is convenient to set up that number of tubes which can be centrifuged simultaneously
2 Add 3 ml of redistilled water to each mix with glass rods and place in boiling water until they become opaque This must be done very carefully or else the solutions will become very gelatinous and coagulation of this type will hinder the extractions which are to follow
3 When the tubes are opaque and still liquid, remove them and cool in a beaker containing cold water
4 Add 2 ml of °0% trichloracetic acid mix thoroughly and break up the precipitate that forms so that it is finely flocculant Then place in water bath at 90 95 C for five minutes
5 Stir this solution carefully and frequently
6 Cool as before, remove the rods and centrifuge the tubes at 3000 r p m for about five to ten minutes
7 Remove and decant the supernatant liquid into a clean dry 15 ml graduated centrifuge tube
8 To the original tubes add ° ml of water and 1 ml of trichloracetic acid being sure to break up the precipitate well by stirring the solution carefully and thoroughly

9 Heat again at 90-95° C for five minutes by carefully stirring the solution to enhance the extraction of the copper
10 Cool again in the cold water centrifuge and decant the solution into the same tubes containing the first filtrate
11 Repeat this operation once again using the same quantities of the water and trichloracetic acid.
12 Make the filtrate up to 15 ml It will contain some precipitate which came over with each decantation
13 Centrifuge this down draw off a 10 ml aliquot and place this aliquot in a Coleman Jr 150 × 19 mm spectrophotometer tube
14 A blank of all the reagents is prepared in the same way This may be prepared directly in the Coleman tube
15 To the filtrate and blank in the Coleman tubes add 1 ml of a saturated solution of sodium pyrophosphate
16 Add 2 ml of ammonium hydroxide solution
17 Add 1 ml of the 0 1% sodium diethyldithiocarbamate solution
18 Add 1 ml of distilled water to bring the total volume up to 15 ml Mix the solutions thoroughly and carefully
19 Set the blank at 100% T (O optical density) and read the unknowns as soon as possible

Calculation

With the Klett set at 100% T copper in mcg per 100 ml serum = (concentration in cuvette) × $\frac{\text{(final dilution)}}{\text{(ml serum)}} \times \frac{\text{(initial dilution)}}{\text{(aliquot)}} \times 100$

Example

5 ml of serum were diluted finally to 15 ml in trichloracetic acid The photometer reading was 9 % T in this case equivalent to 0 3 mcg of copper per ml in the cuvette

Therefore copper mcg per 100 ml serum $= 0.3 \times \frac{15}{5} \times \frac{15}{10} \times 100 = 144$

Precautions

1 Be careful that the solutions do not become gelatinous as this will vitiate results
2 All reagents must be nearly free of heavy metals

Chloride in Serum and Urine

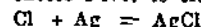
A. CHLORIDE IN SERUM

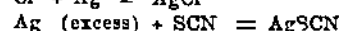
Reference

Keys A The Microdetermination of Chlorides in Biological Materials J Biol Chem 119 389-403 (July) 1937

Principle

The chloride is precipitated as silver chloride with standard silver nitrate and the excess silver is titrated against thiocyanate with ferric alum as indicator


$Cl^- + Ag^+ = AgCl$


$Ag^+ \text{ (excess)} + SCN^- = AgSCN$


$3SCN^- \text{ (excess)} + Fe^{+++} = Fe(SCN)_3$ (RED)

Apparatus

1 A number of round bottom test or centrifuge tubes of 15 ml capacity
A variety of volumetric pipettes including 1 ml 2 ml and 10 ml graduated in 0 1 ml divisions

3 One dropping bottle for storing indicator
4 Water bath (boiling)
5 Assorted bottles, beakers and flasks for reagents and samples
6 Automatic syringe pipettes to deliver 0 1 and 0 2 ml
7 Micro burette, 3 ml

Reagents

For field use it is convenient to carry all reagents except nitric acid weighed out dry in small bottles and to dilute them with distilled water as needed. When many analyses are to be done the best procedure is to have the facilities to make up strong standards ' and to prepare 'working standards'' by diluting the 'strong standard '

1 'Strong ' $AgNO_3$. In a mixing cylinder dissolve 23 785 gm AgNO and dilute to exactly 1 liter with distilled water Tap water may be used if it is chloride free
2 'Working standard' $AgNO_3$. Dilute exactly 100 ml of strong standard to 1 liter with distilled or chloride free tap water
3 'Strong' KCNS Dilute 19 434 gm dry crystals to 1 liter with distilled or chloride free tap water
4 ''Working standard KCNS Dilute exactly 100 ml of ''strong KCNS ' to 1 liter with distilled or chloride free tap water
5 HNO , concentrated, chloride free, reagent grade.
6 Ferric alum indicator Prepare a saturated solution of ferric ammonium sulphate in distilled or chloride free tap water using approximately 40 gm to every 100 ml of water Just before use, add conc HNO_3 drop by drop until the color is yellow not red. This converts ferrous ions to ferric For use, the indicator is stored in a dropping bottle
7 Standard solution of NaCl, 100 mEq/liter Dilute 5 845 gm pure NaCl to exactly 1 liter This reagent is used solely for standardizing the silver nitrate and potassium thiocyanate and keeps indefinitely
8 Distilled or chloride free tap water

Procedure

1 Prepare and store samples of serum as described under the section on handling blood.
2 Deliver exactly 0 2 ml of serum by means of an automatic pipette into a 15 ml test tube
3 Deliver into the same tube 6 to 8 successive washings of the pipette with distilled or chloride free tap water
4 Add exactly 2 0 ml of working standard ' AgNO to each tube
5 Add approximately ¼ ml of conc HNO to each tube Digest on a boiling water bath not less than a half hour More does not matter
6 Cool tubes in ice or cold water bath. This step is essential for a sharp end point
7 Add 3 or more drops of ferric alum indicator
8 Fill the microburette with 'working standard ' KCNS and titrate to the first permanent pink color lasting a half minute or more

Standardization

If the quality of the chemicals is good and weights are accurate the strong ' $AgNO_3$ will be 0 1400 N and the working standard 0 0140 N The strong KCNS will be 0 2000 N and the working standard 0 0200 N This ideal situation rarely occurs and the solutions must be standardized as follows

1 Pipette exactly 5 ml of ''working standard $AgNO_3$ into a small Erlenmeyer flask
2 Add 10 drops of ferric alum indicator

3 Add approximately 1 ml conc HNO_3
4 With working standard KCNS in the burette titrate to the first pink color lasting 1 minute
5 Repeat until satisfactory triplicate estimations are obtained
6 Run the standard solution of sodium chloride exactly as for serum until satisfactory triplicates are obtained
7 The normalities of the working standards of $AgNO_3$ and KCNS may be calculated from the two equations

$$\text{(a) Normality } AgNO_3 = \frac{\text{Titration in step 5}}{5} \times \text{Normality KCNS}$$

(b) Normality NaCl =

$$\frac{(\text{Normality } AgNO_3) - (\text{ml step 6} \times \text{Normality KCNS})}{0\ 00}$$

8 With the known values substituted equations (a) and (b) may be solved as simultaneous equations
9 An example of standardization is

Concentration of NaCl standard	= 0 100 N
Calibration of syringe pipette	= 0 00 ml
Titration in Step 5	= 3 66 ml
Titration in Step 6	= 0 440 ml

Let normality of $AgNO_3$ = ($AgNO_3$) and
normality of KCNS = (KCNS)

$$\text{(a)} \quad (AgNO_3) = \frac{3\ 66}{5} \times (\text{KCNS}) = 0\ 73^{\circ}\ (\text{KCNS})$$

$$\text{(b)} \quad 0\ 100 = \frac{2\ (AgNO_3) - 0\ 440\ (\text{KCNS})}{0\ 00}$$

$0\ 0\ 0 = {}^{\circ}(AgNO_3) - 0\ 440\ (\text{KCNS})$
Substituting from (a) above
$0\ 0\ 0 = \ \times 0\ \iota 3\ (\text{KCNS}) - 0\ 440\ (\text{KCNS})$
$0\ 0\ 0 = 1\ 0 \ldots (\text{KCNS})$

$$\text{and } (\text{KCNS}) = \frac{0\ 0^{\circ}0}{1\ 02^{\circ}} = 0\ 0195$$

From (a) above $(AgNO_3) = (0\ 73^{\circ}) \times (0\ 0195) = 0\ 0143$

Calculation

1 In the case of serum mEq chloride/liter serum =

$$\frac{(2 \times \text{Normality } AgNO_3) - (\text{ml titration} \times \text{Normality KCNS})}{\text{Calibration of Syringe Pipette in ml}} \times 1000$$

2 Line charts are useful for routine calculations when reagents and pipettes are always the same The line chart for *serum* is constructed by marking off the vertical axis according to the titration figure and the horizontal axis for serum chloride Two points only are required with a straight line drawn accurately between. These two points are obtained by substituting all of the figures which are constant in the equation above and then calculating the serum value for titration figures of 0 200 and also 0 500 ml.

For urine set up a chart with the vertical axis titration figure from 0 to 1 500 ml and urinary chloride from 0 to 2 gm sodium chloride per 100 ml as the

horizontal axis The two necessary points are obtained by substituting all of the constant values in the equation above and then calculating for titration figures of 0 and 1 350 ml

Example

Working standard ' AgNO = 0 0139 N
Working standard' KCNS = 0 0234 N
Titration figure = 0 370 ml of KCNS

$$\text{mEq chloride/liter serum} = \frac{(2 \times 0\,0139) - (0\,370 \times 0\,0234)}{0\,200} \times 1000 = 95 \quad \text{mEq/l}$$

Precautions

1 Be sure to titrate with a good daylight or daylight bulb against a white back ground.
2 Do not expose to bright light the silver standards or the test tubes containin unknowns to which silver nitrate has been added.
3 Chloride in serum and urine is of course stable indefinitely at any temperature *Therefore amples may be kept for months before analysis provided there is no* evaporation
4. The operator may leave chloride analyses for hours at any stage provided the tubes containing silver nitrate are not left in the light
5 The titration must be carried out in the cold Oth rwise the results will be er ratic

B CHLORIDE IN URINE

Principle Apparatus Reagents
Same as for serum

Procedure

1 Collect and store samples as described previously
2 Deliver exactly 0 1 ml of urine by means of an automatic syringe pipette into a 15 ml test tube
3 4 5 Steps 3 4 and 5 are identical for urine and serum *except urine need not be di* gested in a boiling water bath unless there is good reason to suspect that the urine contains protein
6 7 8 Steps 6 7 and 8 are identical for urine and serum
9 When the urine contains a high concentration of chloride 2 ml of *working* standard AgNO may not suffice to precipitate all the chloride When this is the case the first drop of thiocyanate produces a red color In such cases add 1 ml more of working standard $AgNO_3$ and proceed with the titration. The titration figure is then the final reading minus the reading before *the first drop* of KCNS

Calculation

1 Grams NaCl/100 ml urine =

$$\frac{(\text{ml AgNO} \times \text{Normality AgNO}) - (\text{ml KCNS} \times \text{Normality KCNS})}{\text{Calibration of syringe pipette in ml}} \times 5\,85$$

2 The use of line charts was described under serum

Example

Working standard AgNO = 0 0139 N
Working standard KCNS = 0 0 05 N
Syringe pipette contains 0 1 ml
3 ml AgNO had to be used

Titration figure 0 550 ml of KCNS

Therefore gm NaCl/100 ml =

$$\frac{(3 \times 0\ 0139) - (0\ 550 \times 0\ 0\ 05)}{0\ 100} \times 5\ 85 = 1\ 78 \text{ gm/100 ml}$$

Precautions

Same as for serum

Inorganic Phosphorus in Serum Urine Food and Feces

N SERUM

Reference

Fiske C H and Subbarow Y The Colorim tric Determination of Phosphorus J Biol Chem 66 375 400 (Dec) 1925

Principle

Inorganic pho phate reacts with molybdate and a blue color is formed by reduc tion with para amino naphthol sulfonic acid

Apparatus

1 Coleman J Spectrophotometer Model #6
Electric centrifuge
3 A series of pipettes including 0 5 ml 5 0 ml and two 10 ml graduated in 0 1 ml divis ons
4 Round bottomed 15 ml centrifuge tubes with #1 rubber stoppers
5 Coleman Jr cuvettes 19 × 150 mm
6 Pan of water and source of heat for water bath
7 An accurately cal brated syringe pipette 1 0 ml
8 A quantity of 50 ml volumetric flasks

Reagents

1 Trichloracetic acid approximately 10% in water
2 Sulfur c acid approximately 10 N In a m xing cylinder carefully pour 100 ml of conc H_2SO_4 into 00 ml of water After cooling dilute to exactly 360 ml with water
3 Molybdate I (for standards and urine) 2 5% ammonium molybdate in 5 N H_2SO_4 In a one liter mix ng cylinder dissolve 25 gm of salt in 500 ml of 10 N H_2SO_4 and dilute to one l ter with water
4 Molybdate II (for serum) 2 5% ammon um molybdate in 3 3 N H_2SO_4 In a one liter mixing cyl nder dissolve 25 gm of the salt in 300 ml of 10 N H_2SO_4 and d lute to one liter with water
5 Para aminonaphtholsulfonic acid (ANSA) To a 250 ml mixing cylinder add 0 5 gm ANSA and 195 ml of 15% sodium bisulfite (metabisulfite gives the same re sults) Add 5 ml of 20% sodium sulfite Mix If the ANSA does not dissolve within a half hour add more sulfite 5 ml at a time until solut on is complete Store n brown bottle It keeps for about 2 weeks

(If the dry ANSA is more than slightly colored brown or purple it must be recrystallized as follows and seve al t mes if necessary

In approximately one l ter of water at about 90 C dissolve 150 gm of sodium bi sulfite and 10 gm of sodium sulfite Add about 30 gm ANSA and stand 15 min utes w th intermittent shaking Filter by suction while still hot Cool the filtrate and add 10 ml of concentrated HCl Let stand for 10 minutes Filter off the crystals wash once with 300 ml of cold water and twice with 100 ml po tions of 95% alcohol Dry in air in the dark and store in a brown bottle.)

6 Standard phosphate In a two liter mixing cylinder place exactly 0 351 gm pure KH_2PO_4 approximately 20 ml 10 N H_2SO_4 and approximately one liter distilled water When dissolved dilute to exactly two liters This solution keeps indefinitely in refrigerator 1 ml equals 0 04 mg P

Procedure

1 To a 15 ml centrifuge tube add exactly 9 0 ml of 10% trichloracetic acid.
2 Add exactly 1 0 ml serum mix and centrifuge till clear
3 To a cuvette add exactly 5 0 ml of filtrate
4 Add exactly 1 0 ml Molybdate II
5 Then add 0 5 ml ANSA and 3 5 ml of distilled water Stopper and mix Read in 10 minutes
6 Prepare a blank using Molybdate I and set at 100% T at 660 mμ Read unknowns
7 Prepare a standard curve using Molybdate I with 0 01 to 0 05 mg P at 0 01 mg intervals

Calculation

$$\text{mg P/100 ml serum} = \frac{(\text{mg P in cuvette}) \times 10 \times 100}{(\text{ml aliquot}) \times (\text{ml serum})}$$

Example

Sample of serum was 1 ml Aliquot was 5 ml of a 10 ml trichloracetic acid filtrate % T was 41 0 equivalent on the calibration curve to 0 02 mg P in the cuvette

$$\text{mg inorganic P/100 ml serum} = \frac{(0\ 02) \times 10 \times 100}{(5) \times (1)} = 4\ 0 \text{ mg P}$$

Precautions

1. The quality of the ANSA is critical If there is any doubt recrystallize it before using

B IN URINE

Procedure

1 Dilute exactly 1 0 ml of urine to 50 ml with distilled water
2 To a cuvette add 5 0 ml of diluted urine
3 Add 1 0 ml of Molybdate I
4 0 5 ml of ANSA and 3 5 ml of water Stopper and mix
5 Place blank at 100% T and read the unknowns at 660 mμ

C IN FOOD AND FECES

Reference

Official A O A C Method
Corley R C and Denis W The Determination of Calcium in Tissues, Feces and Milk J Biol Chem 66 601 608 (Dec) 19?5

Apparatus

1 Steam bath
2 Crucibles (porcelain or quartz)
3 Muffle furnace at 600 C
4 Coleman Spectrophotometer Model #6
5 Cuvettes 19 x 150 mm

Reagents

1 Standard potassium dihydrogen phosphate solution
Dissolve 0 4394 gm of pure dry KH_2PO_4 in water and make up to a liter 50 ml of this solution is d luted to 200 ml and gives a standard of which 2 ml equals 0 05 mg of P
2 Ammonium molybdate solution
Dissolve 5 gm of ammonium molybdate in 300 ml of water Dilute 75 ml of H_2SO_4 to 200 ml and add to the ammonium molybdate solution.
3 Magnesium nitrate solution
Dissolve 160 gm of MgO in HNO_3 (1 volume conc HNO_3 plus 1 volume water), avoiding excess of the acid add a little MgO in excess boil filter from the excess MgO Fe_2O_3 etc and dilute to 1 liter
4. Hydrochloric acid 1 part plus 4 parts of water

Preparation of Solution

1 To 1 gm of sample in a small crucible add 1 ml of the magnesium nitrate solution and place on steam bath.
2 After a few minutes cautiously add a few drops of HCl taking care that formation of gas bubbles does not push portions of sample over edge of crucible
3 Make or 3 further additions of a few drops of HCl while sample is on bath so that as it approaches dryness there is a tendency for it to char
4 If contents of crucible become so viscous that no further drying may be obtained on bath complete drying on a hot plate Then put on crucible cover transfer to cold muffle and ignite at dull red heat for at least 6 hours (600 C) or until an even gray ash is obta ned (It may be necessary to cool crucible dissolve ash in a little water or alcoholic glycerol evaporate to dryness and return uncovered to muffle for 4 5 hours longer)
5 Cool take up with HCl (1 plus 4) and transfer to 100 ml beaker
6 Add 5 ml of HCl and evaporate to dryness on steam bath to dehydrate SiO_2
7 Moisten residue with ml of HCl add about 50 ml of water heat a few minutes on bath transfer to 100 ml volumetric flask cool immediately make to volume and filter discard ng first portion of filtrate
8 From this point on for feces take 3 ml of dilution and for food take 10 ml of dilution
9 The procedure is the same as for phosphorus in urine steps 2 5

D ALTERNATIVE METHOD FOR SERUM

Reference

Tschopp E and Tschopp E Helv Chim Acta 15 793 809 193

Principle

A blue complex forms upon the interaction of phosphates and para methylamino phenol sulfonic acid. The intensity of the color is measured colorimetrically

Apparatus

1 A quantity of 15 ml conical centrifuge tubes.
2 A quantity of analytical filter paper and funnels to match
3 Coleman Jr Spectrophotometer Model 6
4 Cuvettes 19 × 150 mm.
5 Centrifuge
6 A water bath set at approximately 60 C
7 Syri ge pipette 0 1 1 0 ml and 4 7 ml

Reagents

1 Trichloracetic acid (CCl_3COOH) 10% filtered. Keep in refrigerator
2 Sod um bisulfite ($NaHSO_3$) 0% solution in water

3 Ammonium molybdate (Merck Reagent Grade 81% MoO) in 1 N sulfuric acid. Dissolve 25 gm phosphate free salt in water and 50 ml 10 N H SO Dilute to 500 ml Do not use if blue tinge develops Keep in wax lined soft glass bottle to guard against decomposition

4 Photorex

Di solve in water and dilute to 1 liter
2 0 gm p methyl amino phenol sulfonic acid
200 0 gm sodium bisulfite
10 0 gm sodium sulfite

5 Phosphate standard solution

Dissolve in water and dilute to 1 liter
0 35 gm potassium phosphate monobasic
(pure dry salt) and 10 0 ml of 10 N sulfuric acid
1 ml is equivalent to 0 08 mg P

Procedure

1 To a 15 ml cone tipped centrifuge tube add exactly 1 ml serum and 9 ml tri chloracetic acid
2 Mix by inversion and centrifuge for 5 minutes Filter supernatant fluid through quantitative filter paper
3 Pipette 5 ml of filtrate into a cuvette (preferably graduated at 10 ml)
4 Add 0 1 ml sodium bisulfite and mix
5 Add 0 1 ml Molybdate and mix
6 Add 0 1 ml photorex and mix.
7 Dilute to exactly 10 ml and mix or add 4 7 ml of water
8 Immediately heat for 20 minutes in hot water bath at 60 C After 20 minutes cool the tube in running water to room temperature
9 For a blank make up with water and all reagents as for unknowns. Heat similarly Set at 100% T at 660 mμ (or 635 mμ) Then read the unknowns

Standard Curve

Dilute standard in such a way that 5 ml of solution contains 005 mg through 04 mg of phosphorus

Calculation

From the phosphate standard curve determine value of galvanometer readings for unknowns in mg of phosphate As 5 ml of filtrate is equivalent to 0 5 ml serum multiply by 2 To calculate mg of phosphorus per 100 ml multiply by 100

mg P/100 ml serum = (mg P in cuvette obtained from standard table) × 2 × 100

Example

A reading of 41 0% T was equivalent to 0 02 mg of phosphorus on the calibration chart In the cuvette was the equivalent of 0 5 ml serum (5 ml of a 1 to 10 dilution) Therefore mg P/100 ml serum = (0 02) × 200 = 4 0

Precaution

1 Be sure to heat all tubes immediately at 60 C and for exactly 20 minutes.

11 Sulfur in Urine

A. TOTAL SULFATE IN URINE

Reference

Folin O On Sulphur and Sulphur Determinations J Biol Chem 1 131 159 (Jan) 1906

Principle

The sulfuri acid of the conjugated sulfates is set free by boiling with acid. The total sulfates are precipitated as barium sufate and weighed


SO + BaCl = BaSO + 2Cl

Apparatus

1 Porcelain crucibles
Analytical balance
3 Muffle oven set at 600 C
4 A desiccator for drying crucibles over calcium oxide
5 ?50 ml Erlenmeyer flasks
6 Analytical grade filter paper and funnels

Reagents

1 Barium chlor de (BaCl) 5% in distilled water
" Hydrochloric acid (HCl) concentrated.

Procedure

1 Pipette ?5 or 50 ml of urine into a 250 ml Erlenmeyer flask (Less than 25 ml gives erratic results)
2 Add 5 ml of conc HCl and .0 ml water
3 Cover with watch glass boil g ntly for 0 30 minutes and cool
4 Dilute to approximately 150 ml with water
5 Add 5 ml of BaCl (5%) along the side of the flask
6 Do not shake or disturb but mix gently by tilting the flask back and forth
7 Let stand for 1 hour and filter through analytical grade filter paper
b Wash filter with 50 ml of distilled water and place filter paper plus precipitate into a weighed crucible
9 Dry and ignite in muffle oven at 600 C
10 Cool in a des ccator and weigh

Calculation

1 gm S/liter urine =

$$(\text{gm ppt}) \times \frac{\text{atomic wgt S}}{\text{molecular wgt BaSO}} \times \frac{1000}{\text{ml urine}}$$

$$= (\text{gm ppt}) \times \frac{3\ 06}{33\ 43} \times \frac{1000}{\text{ml u i e}}$$

$$= \frac{\text{gm ppt}}{\text{ml urine}} \times 137\ .$$

Example

1 P ecipitate of $BaSO_4$ 1 . 0 mg
Ur ne analyzed 5 ml

$$\text{gm S/lite urine} = \frac{0\ 1\ \ 1}{5} \times 137\ 5 = 0\ 6?0$$

This computation is facil tated by Table 6

Precautions

1 At least 5 ml of urine have to be used in this method Otherwi e recovery of added sulfate is erratic and usually low

TABLE 6

SULFUR IN URINE

When 25 ml of urine are analyzed, gm S/liter urine = (gm ppt) × 5 5 or (mg ppt) × 0 0055

mg ppt	0	2	4	6	8
	GRAMS SULFUR PER LITER OF URINE				
0	0	01	0[illegible]	03	04
10	06	07	08	09	10
20	11	12	13	14	15
30	17	18	19	20	[illegible]1
40	22	23	24	25	6
50	28	.29	30	31	3[illegible]
60	33	34	35	.36	37
70	39	40	41	42	43
80	44	45	46	47	48
90	.50	51	.5[illegible]	53	[illegible]4
100	55	[illegible]6	57	58	[illegible]9
110	61	62	63	64	65
1[illegible]0	66	67	68	69	70
130	72	73	74	75	76
140	77	78	79	80	81
150	83	84	85	86	87
160	88	89	[illegible]0	91	92
170	94	95	96	97	98
180	99	1 00	1 01	1 02	1 03
190	1 05	1 06	1 07	1 08	1 09
200	1 10	1 11	1 1[illegible]	1 13	1 14
210	1 16	1 17	1 18	1 19	1 20
220	1 21	1.22	1 23	1 24	1 [illegible]5
230	1 27	1 28	1 29	1 30	1 31
240	1 3[illegible]	1.33	1 34	1 35	1 36
250	1 38	1 39	1 40	1 41	1 4[illegible]
260	1 43	1 44	1 45	1 46	1 47
270	1 49	1.50	1 51	1 52	1 53
280	1 54	1.55	1 56	1 57	1 58
290	1 60	1 61	1 6[illegible]	1 63	1 64
300	1 65	1 66	1 67	1 68	1 69
310	1 71	1 72	1 73	1 74	1 75
320	1 [illegible]6	1 77	1 78	1 79	1.80
330	1 82	1 83	1 84	1 85	1 86
340	1 87	1.88	1 89	1 90	1 91

B INORGANIC SULFATE IN URINE

Reference

Folin O On Sulphate and Sulphur Determination J Biol Chem. 1 131 159 (Jan.) 1906

Principle

The inorganic sulfates are precipitated at once as barium sulfate

Apparatus

1 A variety of 250 ml Erlenmeyer flasks
2 Crucibles (porcelain)
3 Desiccator
4. Analytical balance
5 Analytical filter paper
6 Muffle oven—600 C

Reagents

1 Hydrochloric acid dilute One volume to 4 volumes of distilled water
2 Barium chloride 5% solution

Procedure

1 Pipette 5 ml of urine into a 250 ml Erlenmeyer flask
2 Add approximately 100 ml of water
3 Then acidify with 10 ml of hydrochloric acid.
4 Add 10 ml of barium chloride solution slowly drop by drop to the cold solution
5 Allow to stand for one hour and filter
6 Wash the filter paper with 50 ml of water and then place the filter paper plus the precipitate into a weighed crucible
7 Dry and ignite in muffle oven at 500 600 C (overnight)
8 Cool in a desiccator and weigh

Calculation

1 gm S per liter urine =

$$(\text{gm ppt}) \times \frac{\text{atomic wgt S}}{\text{molecular wgt } BaSO} \times \frac{1000}{\text{ml urine}}$$

$$= (\text{gm ppt}) \times \frac{3\ 06}{33\ 43} \times \frac{1000}{\text{ml urine}} = \frac{\text{gm ppt}}{\text{ml urine}} \times 137\ 5$$

Example

Precipitate of BaSO = 105 0 mg
Urine analyzed = 25 ml

$$\text{gm inorganic S per liter urine} = \frac{0\ 1050}{25} \times 13\ 5 = 0\ 578 \text{ gm}$$

(This calculation is facilitated by Table 6)

Precautions

1 At least 25 ml of urine have to be used in this method otherwise recovery of added sulfate is erratic and usually low

ETHEREAL SULFATES

Reference

Folin O On Sulphate and Sulphur Determinat on J Biol Chem 1 131 159 (Jan) 1906

Calculation

Total S – Inorganic S = Ethereal sulfates

Example

0 686 gm – 0 578 = 0 108 gm

2 Minerals Miscellaneous References

Alkaline Earths

allmann S Quantitative Separation of Calcium Barium and Strontium Anal Chem 20 449 451 (May) 1948

Aluminum

trafford N and Wyatt P F The Separation and Determination of Very Small Amounts of Aluminum and Iron in Water Analyst 68 319 3 4 (Nov) 1943
Wolff H Spektrochemische Untersuchungen über den Aluminiumgehalt des Blutes Bio chem Ztschr 319 1 8 No 1 1948

Antimony

Maren T H Colorimetric Micro-Determination of Antimony With Rhodamine B Anal Chem 19 (7) 487-491 (July) 1947

Bromine

Wuth A A Rational Bromide Treatment, J A M A 88 2013 2017 (June) 19 7
Tsyvina, B S Determination of Small Quantities of Calcium Zavodskaya Lab 15 142 144 1949
Tyner E H Determining Small Amounts of Calcium in Plant Materials Anal Chem 20 76 80 (Jan) 1948

Chlorine

Binkley F A Colorimetric Reaction of Chloride Ion J Biol. Chem 173 (1) 403 405 (March) 1948
Ellms J W and Hauser S J Ortho Tolidine as a Reagent for the Colorimetric Estima tion of Small Quantities of Free Chlorine J Indust Engin Chem 5 915 917 (Nov) 1913
Hoffman W S Photoelometric Clinical Chemistry New York, 1941 W Morrow and Com pany pp 129 137
Schales O and Schales S S A Simple and Accurate Method for the Determination of Chloride in Biological Fluids J Biol Chem 140 (3) 879 884 (Sept) 1941
Taylor W A Lamotte Wuth Bromide Comparator J Lab & Clin Med 13 49 496 (Oct March) 19 7 1928
Stiff, H A Jr The Colorimetric Determination of Blood Chloride by the Iodometric Method J Biol Chem 172 (2) 695 698 (Feb) 1948
Swift E H Arcand G M Lutwack R and Meier, D J Determination of Chloride Anal Chem 22 (2) 306 308 (Feb) 1950
Whitehorn J C Simplified Method for the Determination of Chlorides in Blood or Plasma J Biol Chem 45 419-460 (Feb) 1921
Zimmerman W J and Layton E M Jr Polarographic Micromethod for the Determina tion of Blood Chloride J Biol Chem 181 (1) 141 147 (Nov) 1949

Calcium

Alten F Weiland H and Knippenberg E Kolorimetrische Calciumbestimmung uber das Pikrolonat Biochem Ztschr 265 266 85 89 1933
DeLuca H A Photometric Microdetermination of Calcium Canad. J Research 25 B 449 454 (Sept) 1947
Klement R Rapid and Simple Determination of Small Quantities of Calcium Z Anal Chem 128 431 435 1948
Korenman I M and Gutnik G B Determination of Small Quantities of Calcium Zavodskaya Lab 15 136 138 1949
Murnaghan M F Micro Diffusion Method for the Determination of Calcium in Blood Serum Analyst 74 546 549 (Oct) 1949
Ringbom A and Merikanto B A Titrimetric Method for the Determination of Calcium Acta Chem Scandinav 3 29 31 No 1 1949
Roe J H and Kahn B S The Colorimetric Determination of Blood Calcium J Biol Chem 81 1 8 (Jan) 1929
Shohl A T The Effect of Hydrogen Ion Concentration Upon the Determination of Calcium J Biol Chem 50 527 536 (Feb) 19?
Shohl A T, and Pedley F G A Rapid and Accurate Method for Calcium in Urine J Biol. Chem 50 537 544 (Feb) 192?
Sobel A E and Sklersky S A Direct Acidimetric Microtitration Method for Calcium J Biol Chem 122 665 672 (Feb) 1938
Trevan J W and Bainbridg H W The Estimation of Calcium in Blood Serum Bio chem. J 20 4 3 426 No ? 19?6

Cobalt

Claassén A and Westerveld W Photometric Determination of Cobalt With Nitroso R Salt Rec Trav Chim 67 720 724 1948
McNaught K J The Determination of Cobalt in Animal Tissue Modification of the Method Analyst 67 97 98 (March) 194?
Van Klooster H S Nitroso R Salt a New Reagent for the Detection of Cobalt J Am Chem Soc 43 746 749 (Jan) 19?1
Willard H, H and Kaufman S Colorimetric Determination of Cobalt Using Nitroso R Salt Anal Chem 19 505 (July) 1947

Copper

Oglethorpe C C and Smith C G Determination of Copper Volumetrically by the Iodine Thiocyanate Method Analyst 68 3?5 3 8 (Nov) 1943

Iodine

Barker S B Determination of Protein Bound Iodine J Biol Chem 173 715 7 4 (April) 1948

Connor A C Curtis G M and Swenson R E A Simplified Method for the Determination of the Protein Bound Blood Iodine and Its Clinical Application J Clin Endocrinol 9 (11) 1185 1189(Nov) 1949

Dobouloz P Monge Hedde M F and Fondarai J Colorimetric M crodeterm nation of Iodine Bull Soc Chim b ol France pp 898 899 1947

Fine S D Report on Iodine J Assoc Offic Agric Chemists 32 (3) 555 558 1949

Gomberg H J Beierwaltes W H and Lampe I Rad o Iodine Uptake in Humans V Absolute Determination of Radio Iod ne in the Thyro l Proc Soc Exper Biol & Med 73 40 408 (March) 1950

Gross W G Wood L K and McHargue J S Spectrophotometric Determination of Iodine Anal Chem 20 900 901 (Oct) 1948

Lacourt A a d Timmermans A M Contributions to the Volumetric Mirco Method for the D termination of Organ c Iodine Anal Chim Acta 1 (2) 140 148 1947

McCullagh D R A New Method for the Determination of Iodine J Biol Ch m 107 (1) 35 44 (Oct) 1934

Salters W T and McKay E A Iodine in Blood and Thyroid of Man and Small Animals Endocrinol 35 90 3?0 (Nov) 1944

Stimmel B F and McCullagh D R A Note Concerning the Determ nation of Iod ne J B ol Chem 116 (1) 1 4 (Nov) 1936

Iron

Cooke W D Hazel F and McNabb W M Volumetric Determination of Small Amounts of Iron Anal Chem 21 1011 (Aug) 1949

Hahn P F Bale W F Hettig R A Kamen M D and Whipple G H Radio active Iron and Its Excretion in Urine Bile and Feces J Exper Med 70 443 451 (Nov) 1939

Worwod A J The Determination of Iron in Biological Material B ochem J 41 (1) 39 41 1947

Magnesium

Flaschka H Rapid Determination of Calcium and Magnes um Monatsh 80 506 509 1949

Garner R J Colorimetric Determination of Magnesium in Plasma or Serum by Means of Titan Yellow Biochem J 40 (5/6) 8 8 831 1946

Kunkel H O Pearson P B and Schwe gert B S The Photoelectric Determination of Magne um in Body Fluid J Lab & Clin Med 32 (8) 10 7 1033 194

Michaels G D Anderson C T Margen S and Kinsell L W Colorimetric Determina tion of Ca and Magnes um in Small Amounts of Urine Stool and Food J Biol Chem 180 175 180 1949

Manganese

Gate , F M and Ellis G H A Micro Colorimetric Method for the Determination of Manganese in B ological Mater als With 4 4 Tetramethyldiaminotriphenylmethane J Biol Chem 168 3 544 (May) 1947

Kun E Mi rod te m nation of Manganese in Biological Material by Means of Catalysis J Biol Chem 170 509 514 (Oct) 1947

Mixtures

Boyle A J Whitehead T, Bird E. J Batchelor T M. Iseri T Jacobson S D and Myers G B The Use of the Emission Spectrograph for the Quantitative De termination of Na K Ca Mg and Fe in Plasma and Urine J Lab & Clin Med. 34 (5) 6 5 630 1949

Polley J R The Microdetermination of Sodium Potassium Calcium and Chloride in a Single Plasma Sample Canad. J Research 26 E 188 195 (April) 1948

Phosphorus

Br gg A P Some Appl cations of the Colorimetric Phosphate Method J Biol. Chem 59 5 65 (March) 19 4

Kuttner T and Cohen H R Micro Colorimetric Studies I. Molybd c Acid Stannous Chloride Reagent The Micro Estimation of Phosphate and Calcium in Pus Plasma an l Spinal Fluid J Biol Chem. 75 517 53 (N ov) 19 7

Soyenkoff B A Micromethod of Phosphate Determination J Biol. Chem 1 447 457 (May) 1947

Potassium

Breh F, and Gaebler O H The Determination of Potassium in Blood Serum J Biol Chem 87 (1) 81 90 (May) 1930

Chen Y C, and Shen S S Determination of Potassium by Zinc Cobaltinitrite J Chinese Chem Soc 8 (1) 12 14 1941

Dahr E Determination of Potassium in Urine by Means of Periodic Acid Acta physiol Scandinav 12 229 234, 1946

Folch J, and Lauren M Estimation of Potassium in Biological Materials as Potassium Phosphotungstate J Biol Chem 169 (3) 539 549 (Aug) 1947

Harris J E A Modified Silver Cobaltinitrate Method for Potassium Determination J Biol Chem 136 619 627 (Dec) 1940

Looney J M and Dyer C G A Photoelectric Method for the Determination of Potassium in Blood Serum J Lab & Clin Med 28 (3) 355 363 (Dec) 194?

Shohl A T and Bennett H B A Micro Method for the Determination of Potassium as Iodoplatinate J Biol Chem 78 643 65? (Aug) 1928

Van Rysselberge P J Quantitative Determination of Potassium by Sodium Cobaltinitrite Method *Indust & Engin Chem Anal Ed 3 (1) 3 4 (Jan) 1931*

Sodium

Albanese, A A and Lein M The Microcolorimetric Determination of Sodium in Human Biologic Fluids J Lab & Clin Med 33 246 250 (Feb) 1948

Bills C E McDonald F G Neidermeier W and Schwartz M C Sodium and Potassium in Foods and Waters Determination by the Flame Photometer, J Am Dietet A 25 (4) 304 314 (April) 1949

Butler A M and Tuthill E An Application of the Uranyl Zinc Acetate Method for Determination of Sodium in Biological Material J Biol Chem 93 171 180 (Sept) 1931

Hald P M Heinsen A J and Peters J P The Estimation of Serum Sodium From Bicarbonate Plus Chloride J Clin Investigation 26 (5) 983 990 (Sept) 1947

Marinis T P Muirhead F L Jones F and Hill J M Sodium and Potassium Determinations in Health and Disease J Lab & Clin Med 32 (10) 1 08 1 16 (Oct) 1947

McCance R A and Shipp H L A Colorimetric Determination of Sodium Biochem J 25 449 456, No 2 1931

Stone, G C H and Goldzieher J W A Rapid Colorimetric Method for the Determination of Sodium in Biological Fluids and Particularly in Serum J Biol Chem 181 511 522 (Dec) 1949

Weinbach A P A Micromethod for the Determination of Sodium J Biol Chem 110 (1) 95 100 (June) 1935

Willebrands A F Jr Determination of Na and K in Blood Serum and Urine by Means of the Flame Photometer Nederl tijdschr v geneesk 93 III 2351 2356 1949

Sulfur

Erdos J Procedure for the Micro Determination of Sulphur Mikrochemie ver Mikrochim Acta 34 286 288 1949

Kahn B S and Leiboff S L Colorimetric Determination of Inorganic Sulphate in Small Amounts of Urine J Biol Chem 80 623 630 (Dec) 1928

Klimenko V G Microdetermination of Sulphur in Biological Materials Biokhimiya 14 1 4, 1949

Leclerc R Volumetric Determinations of Sulphate Mém Services Chim état 3? 15 19 1945

Letonoff, T V and Reinhold J G A Colorimetric Method for the Determination of Inorganic Sulfate in Serum and Urine *J Biol Chem 114 (1) 147 156 (May) 1936*

Power, M H and Wakefield E G A Volumetric Benzidine Method for the Determination of Inorganic and Ethereal Sulphate in Serum J Biol Chem 123 665 6?8 (May) 1938

Shu Chuan Liang Y W and Tzu Cheng C Gravimetric Determination of Sulphur as *Barium Sulphate II. Elimination of Chromic Ion Interference by Means of Tannin* J Chinese Chem Soc 14 9 16 1946

Sperber I A Direct Turbidimetric Method for Determining Ethereal Sulfates in Urine J Biol Chem 172 (2) 441 444 (Feb) 1948

Steyermark A Bass E and Littman B Microdetermination of Sulfur in Organic Compounds Anal Chem 20 587 (June) 1948

Stockholm M and Koch F C A Quantitative Method for the Determination of *Total* Sulfur in Biological Material J Am. Chem Soc 45 (3) 1953 1959 (Aug) 19 3

Wakefield E G The Colorimetric Determination of Total and *Inorganic Sulphates* in Blood Serum Urine and Other Body Fluids J Biol Chem 81 713 7?1 (March) 19 9

Total Base

Fiske C H. A Method for the Estimation of Total Base in Urine J Biol Chem 51 (1) 55 61 (March) 19?
Stadie W C and Ross E C A Micro Method for the Determination of Base in Blood and Serum and Other Biological Materials J Biol Chem. 65 735 54 (Oct) 19 5

Water

Sapirstein L A A Simple Method for the Densiometric Determination of Heavy Water J Lab & Clin Med 35 793 794 (May) 1950

Zinc

Hoch F L and Vallee B L Precipitation by Trichloracetic Acid as a Simplification in Determination of Zinc in Blood and Its Components J Biol. Chem 181 (1) 295 306 (Nov) 1949
Vallee B L and Gibson J G 2nd An Improved Dithizone Method for the Determinations of Small Quantities of Zinc in Blood and Tissue Samples J Biol Chem 176 435 443 (Oct) 1948

SECTION IV

BIOCHEMICAL PROCEDURES (Continued)

B PROTEIN AND NITROGEN COMPOUNDS

1 Total Nitrogen (Micro Kjeldahl) in Serum Urine, Food and Feces

A. IN SERUM AND URINE

References

Keys A A Rapid Micro Kjeldahl Method, J Biol Chem 132 181 187 (Jan.) 1940

Ma T S and Zuazaga G Micro Kjeldahl Determination of Nitrogen A New Indicator and an Improved Rapid Method, J Ind & Eng Chem (Anal Ed) 14 280 282 (March) 1942

Principle

Serum and urine are digested with a phosphate catalyst plus hot sulfuric acid and converted to ammonium sulfate The ammonia is liberated by the addition of alkali distilled into a boric acid solution and titrated directly with standard acid.

Apparatus

1 Calibrated syringe pipette, 0 2 ml (Krogh type)
2 A quantity of 100 ml Kjeldahl (micro) flasks with standard tapered joints
3 A manifold of six micro burners with a good hood
4 Micro Kjeldahl ammonia apparatus (Procured at Macalaster Bicknell Co Cambridge Mass See Fig 18)
5 A 2 ml burette (Krogh syringe)
6 *A number of 15 ml round bottom test tubes (Rehberg type)*
7 A number of 100 ml volumetric flasks (for food and fecal analyses)
8 Luer lok syringe 5 ml

Reagents

1 Mixed indicator Prepare in 95% alcohol 10 ml bromcresol green (0 1%) and 2 ml methyl red (0 1%) This solution keeps well at room temperature
2 Boric acid 2% (approximately) Stored in a glass stoppered bottle it keeps indefinitely
3. Hydrochloric acid or sulfuric acid 0 3 N accurately titrated
4 Sodium hydroxide 30% (approximately) Store in bottle with rubber stopper Keeps indefinitely
5 Catalyst Powder and mix potassium phosphate, dibasic (K_2HPO_4) and hydrated copper sulfate ($CuSO_4$) in the proportion (approximately) 4 1
6 Concentrated sulfuric acid C P (A C S)
7 Grease for glass joints Use pure glycerol or a mixture of 50 ml glycerol with 5 gm NaOH
8 Standard ammonium sulfate Dilute exactly 2 30 gm of ammonium sulfate to 1000 ml of water This is equivalent to 0 5 mg N/ml

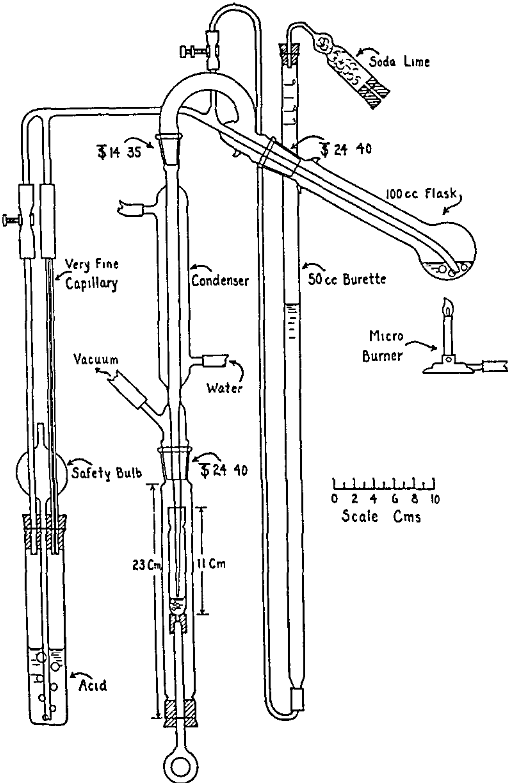


Fig 18.—Still for di till tion of ammonia in a partial vacuum, howing compl te apparatus in use duri g di tillation. (R produced with permissi n of the manufacturers.)

Procedure

1 Pipette exactly 0.2 ml of serum or urine with a syringe pipette into digestion flask, taking care to deliver to the very bottom of the flask Wash out pipette with distilled water 6 times
2 Add roughly 2 gm of catalyst (phosphate mixture)
3 Add approximately 1 5 ml of concentrated sulfuric acid.
4 Digest over burners with exhaust or in hood until solution becomes a clear green Let flasks cool gradually to room temperature
5 Pipette 2 ml of 2% boric acid into collecting tubes (15 ml)
6 Add one drop of mixed indicator to each tube
7 Add approximately 10 0 ml distilled water down side of digestion flask.
8 Distill contents of flask into boric acid contained in Rehberg tube as follows
  a Lubricate mouth of flask with glycerol—NaOH and attach to still
  b Attach Rehberg tube containing boric acid to still so that delivery tube is just below surface of boric acid
  c Turn on vacuum slowly
  d When pressure is uniform throughout still add approximately 6 ml of sodium hydroxide to flask by opening cock under burette
  e Wait 1 to 2 minutes until the evolution of gas decreases (when the rate of bubbling in the acid tube will decrease) and turn on full vacuum
  f Light micro burner under flask with very low flame for the first few minutes gradually increasing the flame until it touches the flask
  g Collect distillate At intervals lower boric acid tube so that delivery tip remains only ½ inch below surface of distillate
  h. Collect 9 10 ml distillate
  i. Pull acid tube b low tip of delivery tube
  j Distill 5 6 more drops and turn off gas flame
  k In 15 20 seconds turn off vacuum
  l Wait for bubbling to quiet down and open capillary cock slowly
  m Allow time for pressure within and without still to equalize
  n Remove collecting Rehberg tube and flask.
  o Remove digestion flask by holding its bulb with a towel Be sure to drain off excess alkaline solution on tip of glass tube which inserts into the flask.
9 Titrate contents of micro tube with 0 3 N H_2SO until solution first turns a distinct orange Use micro burette (2 ml) and air pressure for stirring

Blank

Digest and titrate as above a solution of distilled water with 1 gram digest mixture and 1 ml of acid.

Standard

Place 5 ml of standard ammonium sulfate (NH) SO in a digestion flask and proceed as usual

Calculation

1 Grams N per 100 ml of serum or urine =

$$\frac{0\ 014 \times (\text{ml titration} - \text{ml blank}) \times (\text{Normality of acid}) \times 100}{\text{Volume of sample in ml}}$$

2 To convert nitrogen to protein in serum multiply by the factor of 6 •5

Example

1 Normality of standard acid = 0 335 N

(Equivalent to $14 \times \frac{1}{1000} \times 0\ 335$ gm nitrogen per ml)

2 Amount of serum us d in analyses was 0 231 ml
3 ml of standard acid used in titration was 0 560 ml
4 Blank analysis = 0 02 ml acid.
5 Therefore

$$\frac{014 \times (560 - 0 \ ) \times (0 \quad 5) \times 100}{0\ 231 \text{ ml}} = 1\ 09 \text{ gm nitrogen per 100 ml}$$

6 Then
1 09 gm nitrogen/100 ml × 6 5 = 6 8 gm protein/100 ml

Precautions

1 Never allow the distillate t p to be above the boric acid level
2 In digesting the sample do not allow the sample to dry If necessary add more concentrated sulfuric ac d
3 Pinse out the 0 2 ml syringe pipette at least 6 times with water adding all washings to the flask
4 Be sure that enough alkal is added to liberate the ammon a This can be determined by a change in color from green to blue or brown.
5 The catalyst recommended above serves for most biological fluids If unusual compounds might be present it is necessary to check the efficiency of the catalyst and perhaps to use selenium or mercury as catalyst

B NITROGEN IN FECES AND FOOD

1 Once the food and feces are diluted with water and homogenized in a Waring blendor it is convenient to pipette out 5 ml for digesting
2 We use a cal brated 5 ml Luer Lok syringe for measuring as it has a larger bore than the regular syringe
3 Add 0 ml of concentrated H SO and 4 gm of the catalyst
4 After d gestion to a clear green the flask is cooled and diluted *very slowly* to 100 ml (actually only concentrated H SO is left in the flask so be *very* cautious)
5 Then distill either 10 or 0 ml of solution

2 Serum Proteins (Biuret Method)

Reference

Wolfson W Q Cohn C Calvary E and Ichiba F Studies in Serum Proteins V A Rapid Procedure for the Estimation of Total Protein True Albumin Total Globulin Alpha Globulin Beta Globulin and Gamma Globulin in 1 0 ml of Serum Am J Clin Path 18 , 3 730 (Sept.) 1948

Principle

The albumin and the three major globulin fractions are determined after precipitation and measured colorimetrically in a spectrophotometer by the addition of an alkaline copper sulfate reagent (biuret) which produces a blu sh purple color

Apparatus

1 Cuvettes 19 × 150 mm
2 Coleman Jr Spectrophotometer Model #6
3 Centrifuge tubes—15 ml round bottom
4 Centrifuge tubes—15 ml conical
5 Centrifuge—electric
6 Incubator set at 3 C
7 Calibrated 0 1 0 2 2 4 4 9 and 9 8 ml syringe pipettes.
8 Rubber stoppers #1 solid.

Reagents

1 23% sodium sulfate Exactly 23 grams of anhydrous sodium sulfate in distilled water at 37 C Make up to 100 ml and keep in an incubator at 37 C
2 28% sodium sulfite solution Exactly 28 gm in water at 28 C Shake and dissolve
3 Saline ammonium sulfate In a liter volumetric flask dissolve 193 gm of ammonium sulfate in approximately 500 ml of water Add 40 gm of sodium chloride (NaCl) dissolve and make up to 1000 ml
4 Biuret reagent (Weichselbaum T E *Am J Clin Path 16 Tech. Sec* 10 40 49 1946) Dissolve 90 gm of Rochelle salts in about 400 ml of 0 2 N NaOH. Add 10 gm of $CuSO_4 5H_2O$ When dissolved add 10 gm of potassium iodide and make up to 2000 ml with 0 2 N NaOH Store in rubber stoppered waxed glass bottle
5 Span ether reagent (We are indebted to the Atlas Powder Co of Wilmington Delaware for Span)
1 ml Span – 20 }
99 ml Ether (USP) } Filter through fast paper and dilute to 100 ml with ether
6 Ether USP

Procedure for Total Protein

For a standard curve use saline as the diluent and use protein concentrations of 1 0 to 8 0 gm per 100 ml at intervals of one gram Use serum albumin concentration of 25 gm per 100 ml

1 Pipette exactly 0 1 ml of serum into a 15 ml round bottom tube and dilute to 5 ml with 4 9 ml of distilled water Mix well by inversion
2 Transfer exactly 3 ml to a cuvette
3 Add exactly 3 ml of biuret reagent and mix by inverting the tube
4 Prepare a blank with 3 ml of water and 3 ml of biuret reagent Save the same blank for determination of albumin plus alpha globulin and for gamma globulin.
5 Let stand for at least 30 minutes and read at 540 mμ placing the blank at 100% T The color is stable for 24 hours

Procedure for Serum Albumin Plus Alpha Globulin

1 Pipette exactly 2 4 ml of sodium sulfate solution into a test tube This solution is always kept in an incubator at 37 C
2 Add exactly 0 1 ml of serum and mix by inversion *Do not shake*
3 Add approximately 1 ml of ether stopper and mix by inversion for 30 seconds Centrifuge for 5 to 10 minutes at 1500 to 2000 r p m
4 *Hold tube on a slant and withdraw exactly 1 5 ml of clear centrifugate* and transfer to a cuvette
5 Add exactly 1 5 ml of distilled water and 3 0 ml of biuret reagent Mix well.
6 Read after 30 minutes at 540 mμ, setting blank at 100% T

Procedure for Serum Albumin

1 Pipette exactly 4 9 ml of sodium sulfite solution into a 15 ml round bottom tube.
2 Add exactly 0 1 ml of serum and mix thoroughly by inversion
3 Add about 1 ml Span ether reagent stopper and invert gently for 30 seconds. *Do not shake* In some cases up to 30% of the albumin is lost by vigorous shaking Centrifuge as above
4 Pipette exactly 3 0 ml of the clear centrifugate into a cuvette
5 Add 3 ml of biuret reagent and mix well by inversion Prepare a blank by usin *3 ml sodium sulfite* plus 3 ml biuret reagent
6 Let stand 30 minutes and read at 540 mμ setting the blank at 100% T

Procedure for Serum Gamma Globulin

1 Pipette exactly 9 8 ml of saline ammonium sulfate solut on into 15 ml conical tube
" Layer exactly 0 2 ml of serum on top of the solution Stopper and mix by careful slow repeated inversion for about a minute or two unt l the turbidity has reached a maximum
3 Remove exactly 1 0 ml of the mixture and discard.
4 Cork and centrifuge at 2 50 to ,50 r p m for 0 minutes If turbid cool under tap water for a few minutes and centrifuge again Supernatant fluid *must* be clear
5 Decant supernatant flu d and centrifuge for 5 minutes more Invert tubes on a layer of paper toweling or filter paper for a few minutes
6 First pipette exactly 3 0 ml of b uret reagent to the precipitate a ld 3 0 ml water and shake br skly for 30 seconds
7 Allow to stand for 15 minutes centrifuge down any slight residual turbidity remaining and decant into a cuvette
8 For the blank use 3 0 ml biuret reagent plus 3 0 ml water Allow to stand 30 minutes and read at 540 mμ with the blank at 100% T
9 Divide the prote n value obtained by 3 to obtain the serum gamma globul n concentration This is the reason for discarding the 1 ml in step 3

Calculation

Serum total globulin = Total prote n − Serum albumin
A/G Ratio = Serum album n ÷ Serum globulin
Serum alpha globulin = (Serum albumin + alpha globul n) − Serum albumin
Serum beta + gamma globulin = Total protein − (albumin + alpha globulin)
Serum beta globul n = (Serum beta + gamma globulin) − Serum gamma globulin

Example

1 *Total protein* A reading of 5 % T on the calibrat on chart is equivalent to 4 16 mg of protein in actual amount In the final analyses we actually used 0 06 ml of serum (3 ml of a 0 1 to 5 0 dilution)
Therefore 4 16 mg = 06 ml of serum
or 6 9 gm of protein per 100 ml of serum.

Albumin A reading of 66% T is equivalent to 2 60 mg of albumin in actual amounts We used 06 ml of serum (3 ml of a 0 1 to 5 ml dilution)
Therefore 6 mg = 06 ml of serum
or 4 3 gm albumin per 100 ml of serum

3 *Alpha globulin plus albumin* A reading of 61% T is equi alent to 3 1 mg of albumin + alpha globulin in actual amounts We used 06 ml of serum (1 5 ml of a 0 1 to 5 ml dilution)
Therefore 3 1° mg = 06 ml of serum
or 5 4 gm of albumin plus alpha globulin per 100 ml of serum

4 *Serum gamma globul n* A reading of 77% T is equivalent to 1.56 mg gamma globulin in actual amounts We used 0 18 ml of serum (9 ml of a 0.2 to 10 ml dilution)
Therefore 1.56 = 18 ml serum
or 0 87 gm of gamma globulin per 100 ml of serum.

5 Total prote n (6 9 gm) − albumin (4 3 gm) = total globulin (6 gm)
Therefore albumin/globulin ratio = 1 6

6 Alpha globulin = (Serum albumin + alpha globulin 5 °4 gm) − albumin (4.3) = 0 94 gm.

7 Beta + gamma globulin = Total protein (6 9) − albumin + alpha globulin (5 °4) = 1 66 gm

8 Beta globul n = b ta + gamma globulin (1 66) − gamma globul n (0 8) = 0 ,9 gm

Precautions

1 In the albumin determination, do *not shake the tube vigorously* In some cases up to 30 per cent of the albumin is lost in this way
2 This method depends on exact pipetting Therefore we have used syringe pipettes throughout the procedure
3 In the serum gamma globulin add the biuret reagent *first* before adding the water
4 The serum albumin must be analyzed by the Kjeldahl method for exact analysis.

3 Albumin and Globulin in Serum

Reference

Howe P E The Use of Sodium Sulfate as the Globulin Precipitant in the Determination of Proteins in Blood J Biol Chem 49 93 107 (Nov) 19 1

Principle

Serum proteins are fractionated by precipitation of globulin in sodium sulfate The albumin in solution is analyzed by one of the total nitrogen methods

Apparatus

1 An incubator set at 37 C
2 Filter paper analytical grade (Whatman No 40)
3 Ammonia still and standard tapered flasks (see total nitrogen analyses)
4 Nitrogen digestion unit
5 An accurately calibrated 0 5 ml syringe pipette
6 A quantity of 50 ml Erlenmeyer flasks
7 A supply of funnels

Reagents

1 Sodium sulfate (Na_2SO) anhydrous a 22% solution in water Keep in 37 C oven
2 Thymol crystals
3 Other reagents are the same as those for *Nonprotein Nitrogen* and *Total Nitrogen.*

Procedure

1 Pipette exactly 0 5 ml of serum and 15 ml of sodium sulfate solution into a 50 ml Erlenmeyer flask.
2 Add 1 crystal of thymol
3 Place in incubator at 37 C for 3 hours
4 Heat a funnel a 50 ml Erlenmeyer flask and filter paper (Whatman No 40) by placing in the same oven
5 Filter solution through heated filter into heated flask to prevent precipitation of sodium sulfate The filtrate contains albumin and nonprotein nitrogen If the filtrate is not clear repeat filtration using the same filter paper
6 Digest exactly 5 ml of the filtrate for nitrogen determination using Keys method
7 Estimate nonprotein nitrogen in the original sample of serum following Daly s method as described in the description of nonprotein nitrogen

Calculation

Total protein	=	(Total N − NPN) × 6 25
Albumin	=	(N in filtrate − NPN) × 6 25
Globulin	=	Total protein − albumin
A/G ratio	=	(Albumin ÷ Globulin)

Example

Total serum nitrogen = 1 05 gm. per 100 ml serum
Nonprotein nitrogen = 40 0 mg per 100 ml serum

Total nitrogen in filtrate = 0 67 gm per 100 ml serum
Total protein = (1 05 − 0 04) × 6 25 = 6 32 gm/100 ml
Albumin = (0 67 − 0 04) × 6 25 = 3 94 gm/100 ml
Globulin = 6 32 − 3 94 = 2.38 gm/100 ml
A/G ratio = 3 94 ÷ 2 38 = 1 66

Precautions

1 Time and temperature are critical. Be sure to incubate for exactly 3 hours at exactly 37 C
2 Be sure to have filter paper funnel and flask at exactly 37 C

Whole Blood Hemoglobin and Serum Protein (Copper Sulfate Specific Gravity)

References

Phillips R A Van Slyke D D Hamilton P B Dole V P Emerson K. Jr and Archibald R M Measurement of the Specific Gravities of Whole Blood and Plasma by Standard Copper Sulphate Solutions J Biol Chem. 183 305 330 (March) 1950
Van Slyke D D Hiller A Phillips R A Hamilton P B, Dole V P Archibald R M and Eder H A The Estimation of Plasma Protein Concentration from Plasma Specific Gravity J Biol Chem 183 331 348 (March) 1950
Van Slyke D D Phillips R A Dole V P Hamilton P B Archibald R M and Plazin J Calculation of Hemoglobin from Blood Specific Gravities J Biol Chem 183 349 360 (March) 1950

Principle

The concentration of protein in serum and hemoglobin in cells determines in large part their specific gravities the specific grav ty of whole blood is accounted for by that of serum and cells The present method estimates specific gravity by the behavior of drops of serum and whole blood in solutions of copper sulfate of various specific gravity The serum protein and whole blood hemoglobin are then calculated or more s mply read off from a line chart

Apparatus

1 30 square oz bottles of copper sulfate solution from sp gr 1 0?1 through 1 033 and 1 05 through 1 068 These bottles should have their sp gr etched clearly on them
Four 0 5 ml tuberculin syringes.
3 Four hypodermic needles #?? or finer
4 Four stylets for cleaning needles.
5 Heparinized vials (8 ml) for coll cting whole blood.

Reagents

1 Copper sulfate solid $CuSO_4 \cdot 5H_2O$ finely granular or powdered
a. Copper sulfate solution sp gr 1 100 Allow 1 liter for every 500 samples of blood It is most conveniently carried in large amounts from the base laboratory into the field but can be made up as follows Four pounds of copper sulfate are placed in a gallon jug About 2.5 liters distilled or rain water are added (Tap water may be used if it is not more than sp gr 1 003 compared with distilled water as 1 000 at the same temperature Use urinometer for this comparison.) The bottle is stoppered and shaken vigorously for 5 minutes at

the end of which time the temperature is recorded to the nearest ½ degree C. The saturated supernatant *solution is immediately decanted* and is filtered through cotton into a clean dry jug The solution of sp gr 1 100 is now made at once by diluting accurately to 1 liter the amount of saturated solution indicated in Table 7 Once made the solution of sp gr 1 100 keeps indefinitely Prepare 4 liters at a time

TABLE 7

ML OF SATURATED COPPER SULFATE SOLUTION TO BE DILUTED TO 1 LITER TO MAKE STOCK SOLUTION OF SPECIFIC GRAVITY 1 100

Temperature in C or F refers to the temperature of the saturated solution at the time of saturation (end of shaking for 5 minutes)

TEMPERATURE			TEMPERATURE			TEMPERATURE		
C	F	ml	C	F	ml	C	F	ml
10 0	50 0	578	20 0	68 0	488	30 0	86 0	4 5
10 5	50 9	573	0 5	68 9	484	30 5	86 9	4 3
11 0	51 8	568	21 0	69 8	480	31 0	87 8	4 0
11 5	7	563	21 5	70 7	477	31 5	88	417
1 0	53 6	558	0	71 6	473	3 0	89 6	414
1 5	54 5	553	5	7 5	469	3 5	90 5	41
13 0	55 4	548	23 0	73 4	466	33 0	91 4	409
13 5	56 3	543	23 5	74 3	463	33 5	9 3	406
14 0	5	53	4 0		460	34 0	93	403
14 5	58 1	534	24 5	76 1	456	34 5	94 1	401
15 0	59 0	529	5 0	77 0	453	3 0	95 0	3 8
15 5	59 9	5 5	5 5	77 9	450	3 5	95 9	395
16 0	60 8	5 1	6 0	8 8	447	36 0	96 8	39
16 5	61 7	516	26 5	79 7	445	36 5	97 7	390
17 0	62 6	512	27 0	80 6	44	37 0	98 6	38
17 5	63 5	508	27 5	81 5	439	37 5	99 5	384
18 0	64 4	504	8 0	8 4	436	38 0	100 4	381
18 5	6 3	500	8 5	83 3	434	38 5	101 3	3
19 0	66	496	9 0	84 2	431	39 0	10	3 6
19 5	67 1	492	9 5	85 1	4 8	39 5	103 1	3 3

b Copper sulfate sp gr 1 100 An alternative to method 2 a is to dissolve exactly 170 gm of pure CuSO 5H O in water Amounts of water recommmended for preparing the solution sp gr 1 100

Temperature C	*ml of Water*
10 17 C	1004
17 22 C	1005
26 C	1006
6 29 C	1007
29 33 C	1008
33 36 C	1009
36 8 C	1010
38 40 C	1011

3 Copper sulfate solutions These solutions are most conveniently made according to Table 8 by running the stock solution of sp gr 1 100 from a burette into a 50 ml graduated mixing cylinder, diluting to the mark with water mixing *adding* to the appropriate bottle rinsing the cylinder and *starting over* These solutions should be changed when 50 drops of whole blood or serum have been added It is therefore advisable to make up in the field about 500 ml each of those most commonly used

Procedure

1 The amounts needed are about 0 5 ml each of whole blood and serum prepared as described in a previous section The whole blood must be mixed thoroughly by invers on of its container immed ately before use The whole blood serum and solutions of copper sulfate must all be at approximately the same temperature

TABLE 8

L OF STOCK COPPER SULFATE SOLUTION OF GRAVITY 1 1000 TO BE DILUTED TO 50 ML TO PREPARE STANDARD SOLUTIONS OF GRAVITY G TO WITHIN 0 0001

SP GR	CuSO IN MIXING CYLINDER ML	SP GR	CuSO IN MIXING CYLINDER ML
1 008	3 67	1 043	1 00
9	4 16	44	21 50
10	4 66	45	00
11	5 15	46	[illegible] 50
1	[illegible] 6	47	[illegible]3 00
13	6 14	48	23 50
14	6 64	49	24 00
15	7 13	50	24 50
16	7 63	51	5 00
17	8 1	5[illegible]	5 50
18	8 6	53	6 00
19	9 11	54	6 50
0	9 61	5[illegible]	[illegible]7 00
1	10 10	56	7 50
	10 60	57	8 00
3	11 09	58	28 50
[illegible]4	11 8	59	9 00
[illegible]	1 0	60	9 50
6	1 5	61	30 00
7	13 0[illegible]	6	30 50
[illegible]8	13 54	63	31 00
[illegible]9	14 03	64	31 50
30	14 5	65	3 00
31	1 01	66	3 50
3	15 50	67	33 00
33	16 00	68	33 5
34	16 50	69	34 04
35	17 00	0	34 6
36	1 50	1	35 08
37	18 00		35 60
38	18 50	73	36 1
39	19 00	4	36 64
40	19 50	75	37 15
41	0 00		
4	0 50		

2 For whole blood the range 1 05 thro gh 1 068 is used for serum the range 1 0 1 through 1 033 Fill a syringe with its needle attached to about the 0 [illegible] ml mark If the syringe has just been used for a preceding estimation empty it so far as practicable by pulling the plunger back and forth several times before finally filling the syr nge with the new sample

3 With the copper sulfate rack in a convenient po ition remove the stopper from sp gr 1 060 for whole blood and 1 0 [illegible] for serum Hold the syringe in an al most vertical po ition with the tip of the needle about one half inch above the solution.

LINE CHART FOR CALCULATING PLASMA PROTEINS, HEMOGLOBIN AND HEMATOCRIT FROM GRAVITIES OF PLASMA AND BLOOD

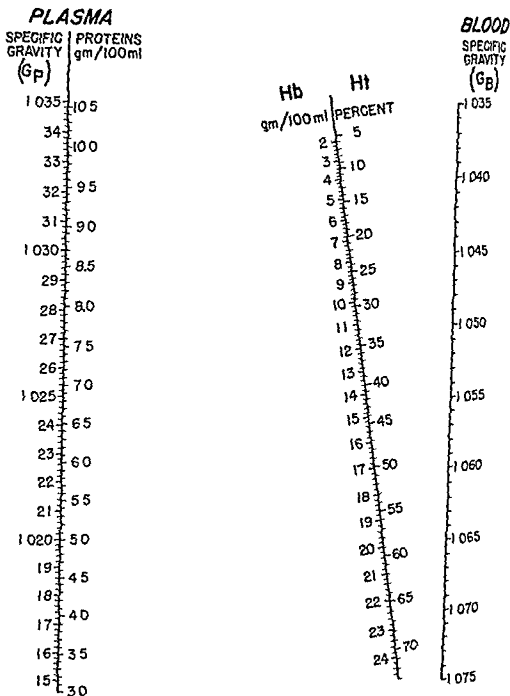


Fig 19—(*From* Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma by Robert A Phillips, Donald D VanSlyke, Vincent P Dole, Kendall Emerson Jr, Paul B Hamilton, Reginald M Archibald. (Reproduced by permission of the author)

4. Deliver a drop from the syringe keeping steady pre sure on the plunger For sat isfactory estimation the drop must break the surface and fall at least an inch into the solution without leaving a streamer attached to the surface
5. Watch the progress of the drop in the solution When its sp gr is exactly the same as that of the solution the drop will fall for a few seconds come to a com plete halt w thout moving up or down for about 10 seconds and will then slowly fall to the bottom because it will take up copper and become heavy When its sp gr is greater than that of the solut on the drop will continue to fall to the bottom If the drop is lighter than the solut on it w ll fall for a few seconds halt for a second or two and begin to rise It will continue to rise until it takes up enough copper to fall to the bottom. If a drop rises on one bottle and falls in the next lightest the sp gr lies between those of the two bottles and must be es timated to the nearest 0 000 by noting carefully the relative rates of rise in one bottle and fall in the next
6. Record the sp gr to the nearest 0 000_ and proceed to the next sample

Calculations

Calculate serum protein in grams per 100 ml and hemoglobin in grams per 100 ml by referring to the l ne chart F g 19

To find plasma protein use the two scales on the left hand line (Note that for plasma the scales read from bottom to top and for the oth rs from top to bottom) To find hemoglobin or hematocrit from G_a and G stretch thread between the num bers on the two outside lines and read the answer on the ins de line To approx imate hemoglobin or hematocrit from G alone stretch the thread between observed G and mean normal G and read from the m ddle line The ranges for no mal values are as follows

	Men	Women
Whole blood specific gravity	1 05 5 to 1 0635	1 05 3 to 1 0603
Plasma specific gravity	1 0 40 to 1 0 80	1 0 40 to 1 0 80

When oxalate is used for an anticoagulant a correction must be made For each mg of 3 ammon um oxalate potas ium o alate added per ml of blood deduct 0 0004 from both G and G

Example

Serum sp gr = 1 0 64
Whole blood sp gr = 1 0_9_
From line chart serum p otein = 7 35 gm per 100 ml and
hemoglobin = 15 9 gm per 100 ml

Precautions

1. The samples and bottles of copper sulfate must be at approx mately the same tempe ature otherwise very serious erro s can be made
2. Thorough mixing of the whole blood is es ential
3. When particles remain on the surface or when the drops fa l to break the surface cleanly the surface should be cleaned with a wooden applicator
4. In the absence of tubercul n syringes ordinary medic ne d oppers can be used
5. The ranges of specific gravity sugge ted above are for normal soldiers in whom hypoprot nemia and anemia are extremely rare For other populations other ranges m ght be neces ary
6. The calculations for hemoglobin and hematocrit are valid only f r normochrom c normocytic blood

EQUATIONS ON WHICH THE LINE CHARTS ARE BASED

(This is copied from the revised method, Don Baxter, Inc, Glendale California whose permission has been given to reprint it here)

The equations given below may be used in place of the line charts to calculate results

Total plasma protein concentration from plasma specific gravity The equation used is

$$(1)\ P = 369\ (G_P - 1.0065)$$

P is the plasma protein concentration in grams per 100 ml G_P is the specific gravity of the plasma 1.0065 is the gravity of protein free plasma ultrafiltrate. In this equation as formerly used in the literature and in previous publications of the copper sulfate method the constant 343 was used instead of the present 369 The value 343 was originally derived by Moore and Van Slyke from observations on nephritic patients for use with such patients

Hematocrit values We have used the formulation of Ashworth and Tigertt for calculating hematocrit values from gravities Let G_B G_P and G_C represent the specific gravities of blood plasma and centrifuged cells, respectively and H the hematocrit number in terms of ml of cells per 100 ml of blood Then the weight of 100 ml of blood 100 G_B can be equated to the sum of weights of its cells and plasma as follows

$$(2)\ 100\ G_B = H\ G_C + (100 - H)\ G_P$$

Rearrangement of Equation 2 to calculate H gives

$$(3)\ H = 100 \times \frac{G_B - G_P}{G_C - G_P}$$

Ashworth and Tigertt pointed out that if cell gravity in different bloods is nearly constant one can substitute its mean value for G_C in Equation 3 and use the latter to estimate hematocrit values from observed G_B and G_P values

To estimate the value of G from observed G G_P and H Equation 3 is rearranged to give Equation 4

$$(4)\ G_C = \frac{100\ (G \quad G) + H\ G}{H}$$

Ashworth and Adams measured in a series of normal and pathological bloods the values of G_B G_P and H, and calculated G_C by *Equation 4 They thus* found the mean value of G_C to be 1.0971 We have made *similar observations in* a series of 20 normal bloods and have obtained a mean G_C of 1.0970 identical *with the value found by Ashworth and Adams* Substituting this value for G_C in Equation 3 gives

$$(5)\ H = 100 \times \frac{G_B - G}{1.0970 - G_P}$$

Equation 5 was used to calculate the hematocrit scale

In the experiments both of Ashworth and Adams and of the *writers* the hematocrit values used in Equation 4 to calculate G_C were obtained by the usual conventional technique for clinical hematocrits using Wintrobe tubes heparinized blood *was centrifuged at 3000 r p m for 60 minutes the center of the centrifuge tube being 18 centimeters from the axis*[1]

When a centrifuge was used in which the centers of the tubes were only 9 cm. from the axis 3000 r p m for an hour gave 51 instead of 4 as the mean

Apparatus

1 Special graduated centrifuge tube (6 5 ml)
2 Electric centrifuge
3 Accurate stopwatch

Reagents

1 Tsuchiya s solution Mix 15 gm of phosphotungstic acid 50 ml of concentrated hydrochloric acid (HCl) and 1000 ml of 95% ethyl alcohol

Procedure

1 Pipette exactly 4 0 ml urine into a special centrifuge tube that is calibrated to 6 5 ml
2 Add 2 5 ml of phosphotungstic reagent
3 Mix several times by inversion and allow to stand *exactly* 10 minutes
4 Centrifuge for exactly 10 minutes at 1800 r p m and read amount of precipitate in terms of ml

Calculation

gram protein/liter = ml of precipitate × 7 2 × dilution (if any)

Example

Urine diluted 1 to 10
Reading 0 11 ml

gram protein/liter = $(0\ 11) \times (7\ 2) \times \frac{(10)}{1} = 7\ 9$

Precautions

1 The timing is very important exactly 10 minutes being required
2 With urine from patients with nephritis it may be necessary to dilute the urine before analysis A dilution of 1 to 10 is usually satisfactory

6 Fibrinogen in Plasma

Reference

Butler, A M and Montgomery H The Solubility of the Plasma Proteins J Biol Chem 99 173 195 (D c) 1932

Principle

Fibrinogen is precipitated in a phosphate buffer and the supernatant fluid is analyzed for nitrogen Fibrinogen is calculated by difference

Apparatus

1 A quantity of 25 ml flasks or test tubes
2 Rubber stoppers
3 Analytical grade filter paper
4 Funnels
5 Apparatus for micro Kjeldahl determinations (see total nitrogen)

Reagents

1 Phosphate buffer 3 0 molar pH 6 5 Into a one liter flask put 408 5 gm of monobasic potassium phosphate (KH_2PO_4) add 375 ml of accurate 4 N potassium hydroxide (KOH) and dilute to 1 liter with water
2 Other reagents are the same as those for the total nitrogen method.

Procedure

1 To exactly 15 ml of phosphate buffer add 1 ml of plasma from citrated blood
2 Mix stopper Let stand overnight at room temperature

3 Filter through good analytical filter paper
4. Analyze a 3 ml al quot for nitrogen by the micro Kjeldahl method
5 Determ ne total n trogen of the plasma by the m cro Kjeldahl method

lculation

Fibrinogen = (total nitrogen − soluble nitrogen) × 6 °5

.ample

Total nitrogen = 1 05 gm per 100 ml plasma
Soluble nitrogen = 0 98 gm per 100 ml plasma
grams fibrinogen per 100 ml plasma = (1 0 − 0 98) × 6 25 = 0 44

ecautions

1 The pH of the precipitating fluid is critical It should be 6 5
° Be sure that the blood s c trated (0 5 ml of saturated sod um citrate per 100 ml of blood)

nprotein Nitrogen in Serum and Whole Blood

ference

Daly C A The Determination of Non Protein Nitrogen With Special Peference to the Koch McMeekin Method J Lab Clin Med 18 1 79 1285 (Sept) 1933

inciple

Combined nitrogen is converte l to ammonia which forms a yellow color in the presence of Nessler s solution

pparatus

1 Coleman Jr Spectrophotometer Model 6
A variety of 15 ml round bottomed centrifuge tubes with rubber stoppers (#1 solid)
3 Electric centrifuge
4 Micro burners in the laboratory or gasol ne stove in the field
5 Folin digestion tubes graduated at 1 5 and 5 ml
6 Glass beads for digestion These should be about mm in diameter
7 A variety of pipettes including 0 5 ml 1 0 ml 5 0 ml graduated in 0 1 ml and 10 ml graduated in 0 1 ml
8 Cuvettes 19 × 150 mm

eagents

1 Nitrogen standard Exactly 0 236 gm of dry ammonium sulfate is made up to exactly one liter with distilled water One ml of this contains 0 05 mg N It keeps for weeks in the cold.
Trichloracetic acid approximately 8 gm in 100 ml water It keeps for at least ° weeks in the cold.
3 Digestion mixture A stock concentrated solution is prepared by pouring *slowly and ca efully with cool g* 2.. ml of concentrated nitrogen free sulfuri acid into 45 ml of water The working mixture is prepare l by diluting exactly 31 ml of the stock solution to 100 ml with distilled water
4 Hydrogen peroxide. Superoxol (30% H O) is diluted with distille l water in the proportion 1 to 10 This 3% H O keeps for a week in the cold
5 Gum ghatti. Approximately 1 gm is di..olved in 100 ml of water and the solution is filtered through cotton
6 Nessler s solut on (after Koch) In a ° liter mixing cylinder place 40 gm of mercuric iodide °0 ml of water and .0 gm of potassium iod de Mix until di. olved and dilute to 00 ml. Add with mixing 27 ml of 10% sodium citrate followed by

975 ml of exactly 10% sodium hydroxide After the solution has cooled to room temperature dilute to exactly 1600 ml with water and store in a Pyrex vessel. It keeps indefinitely but does throw down a deposit

Procedure

1 Precipitate protein by adding exactly 0 5 ml of blood or serum to exactly 4.5 ml of 8% trichloracetic acid in a 15 ml centrifuge tube Stopper and mix by inversion Do not shake or foaming will be annoying
2 Centrifuge at high speed for 5 minutes If necessary filter to take out particles
3 To a digestion tube add exactly 1 0 ml filtrate 1 0 ml of digestion mixture and 2 small glass beads
4 Place the digestion tube in a slanting position directly over a free flame When boiling commences add exactly 2 drops of 3% H_2O_2 (Superoxol usually has some combined nitrogen in it and therefore the exact amount of H_2O_2 is important) During digestion the fluid should turn brown and then become clear At the end dense white fumes of SO_3 will appear in the middle part of the tube
5 Cool in the air Inspect the digest to be sure there is no brown in it, and if there is, redigest
6 At the same time as steps 3 4 and 5 set up a blank tube containing 1 ml of water and a standard tube containing exactly 1 ml (= 0 050 mg N) of standard and treat them exactly like the unknowns
7 Add approximately 10 ml of water and the 0 5 ml of gum ghatti solution
8 Dilute to exactly 1° 5 ml with water
9 Add exactly 5 ml of Nessler s solution with continuous mixing
10 Set spectrophotometer at 5.0 mμ with blank at 100% T and read unknowns.
11 Make up a standard curve using a range of 0 01 mg to 0 07 mg N at intervals of 0 01 mg preferably from standards run through the whole method

Calculations

1 mg nonprotein N/100 ml serum =

$$\frac{(\text{mg N in cuvette}) \times 5 \times 100}{(\text{ml filtrate}) \times (\text{ml serum})}$$

Example

1 Unknown read 74 5% T equivalent to 0 03 mg N in cuvette Serum was diluted 0 5 to 5 0 with trichloracetic acid Of the filtrate 1 ml was used for analysis

$$\text{mg nonprotein N/100 ml serum} = \frac{(0\ 03) \times 5 \times 100}{(1\ 0) \times 0\ 5} = 30\ 0 \text{ mg N}$$

2 Since all samples of serum and blood are treated always alike, it is a saving of time to set up the standard curve in the manner shown in Fig 20 and to read the final concentration directly

Precautions

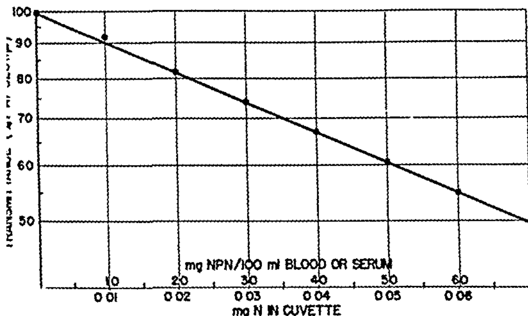
1 Both Nessler s solution and gum ghatti deposit sediments on standing for several days Hence before each day s run it is desirable to decant off enough of the clear supernatant fluid from each for the analyses in prospect
2 The commonest source of error is splattering during digestion This can be avoided by keeping the tubes in gentle motion until boiling starts

8 Creatinine in Urine

Reference

Folin O and Wu H A System of Blood Analysis J Biol Chem. 38 93 100 (March) 1919

NON-PROTEIN NITROGEN IN SERUM AND BLOOD



%T VS NPN — DIRECT CONVERSION

% T	0	1	2	3	4	5	6	7	8	9
	Mg NPN/100 ml Blood or Serum									
50	70	68	66	64	62	60	58.5	57	55	53
60	51.5	50	48	46.5	45	43	42	40.5	39	37
70	36	34.5	33	31.5	30	28.5	27.5	26	25	23.5
80	22	21	20	18.5	17	16	15	13.5	12.5	12
90	10	9	8	7	6	5	4	2.5	1.5	0.5
	NORMAL. 25 to 35 mg/100 ml									

C.E. GORDON

Fig 3.—Use of calibration curve and conversion table for facilitating computation of results in estimating nonprotein nitrogen. The same format is useful for all kinds of colorimetric procedures.

Principle

Creatinine reacts with picrate in alkaline solutions at room temperature and in a few minutes a stable intense orange color is produced which can be measured in a photocolorimeter

Apparatus

1 Coleman Jr Spectrophotometer Model 6
2 Cuvettes 19 × 150 mm
3 100 ml volumetric flasks
4 Calibrated syringe pipettes 0 1 ml and 10 ml
5 Volumetric pipettes of 20 ml capacity

Reagents

1 Sodium hydroxide, a 10% solution in water
2 Picric acid a 1% olution in water Gentle heating may be required to dissolve the acid Mix 100 ml NaOH and 100 ml picric acid and dilute to 1000 ml. This solution is called the alkaline picrate solution
3 Crystalline creatinine zinc chloride Dissolve and dilute 1 6106 gm to 1000 ml in 0 1 N hydrochloric acid This solution contains 1 0 mg of creatinine per ml

Procedure

1 Into a 100 ml volumetric flask measure accurately 0 1 ml of urine with a syringe pipette
2 Dilute to approximately 0 ml with distilled water and add exactly 20 ml of the mixed sodium hydroxide and picric acid mixture
3 Allow to stand for exactly 15 minutes after a gentle mixing and then dilute to 100 ml mark
4 A blank using exactly 20 ml of the alkaline picrate mixture is set up and diluted to 100 ml
5 An aliquot (10 ml) of the blank is pipetted into a cuvette and set at 100% T at a wave length of 5 0 mμ
6 Then read aliquots of the unknowns

Calculation

1 Prepare a standard curve from creatinine zinc chloride solution
   a Dilute exactly 5 ml of standard to 100 ml This gives a solution of 0 05 mg creatinine per ml
   b Into 100 ml volumetric flasks pipette 0 1 2 3 4 and 5 ml of the dilute standard
   c Add 20 19 18 17 16 and 15 ml of wat r respectively
   d Add exactly 20 ml of the same alkaline picrate used for analysis of unknowns
   e Stand exactly 15 minutes and then dilute to 100 ml
   f Set blank at 100% T and read all standards at 5 0 mμ
   g Plot the C %T curve on semi log paper

2 gm creatinine/ liter urine = $\frac{\text{(mg creatinine/ml of final solution)} \times 100 \times 1000}{(1000) \times (0\ 1)}$ =
   (mg creatinine/ml final solution) × 1000

Example

A reading of 81 0% T was equivalent to 0 0009 mg/ml of final solution Therefore gm creatinine/l urine = 0 0009 × 1000 = 0 9 gm/l

Precautions

1 Many variables affect the intensity of the creatinine picrate Important among these are time of standing temperature concentration of alkali and presence of noncreatinine color producing substances Therefore it is essential to establish standard conditions and to adhere strictly to these

9 Creatine in Urine

Reference

Folin O and Wu H A System of Blood Analysis J Biol Chem 38 98 100 (March) 1919

Principle

Creatine is converted to creatinine when boiled or autoclaved with acid. The creatinine is determined before and after the boiling treatment and creatine originally present in the urine is calculated by difference

Apparatus

1 Coleman Jr Spectrophotometer Model 6
   Cuvettes 19 150 mm
3 *A quantity of 100 ml volumetric flasks*
4 Calibrated syringe pipettes 0 1 ml and 10 ml
5 Autoclave set at 15 pounds pressure
6 *A quantity of volumetric pipettes of 10 and 0 ml capacity*

Reagents

1 Sodium hydroxide a 10% solution
   Picric acid a 1% solution in water It may be necessary to warm this to obtain a clear solution
3 *Crystalline creatinine zinc chloride The same concentration as for creatinine* (1 mg/ml)
4 Hydrochloric acid 1 N 10 ml of concentrated HCl plus 100 ml of distilled water

Procedure

1 Into a 100 ml volumetric flask measure accurately 0 1 ml of urine with a syringe pipette
   Add exactly 10 ml of picric acid solution approximately 0 ml of water and ml of HCl solution
3 Autoclave at 15 pounds pressure at a temperature of 115 to 1 0 C for 30 minutes
4 *Allow to cool to room temperature*
5 Add exactly 10 ml of sodium hydroxide and dilute to 100 ml after exactly 15 minutes of standing
6 Run a blank using 10 ml of picric acid solution and 0 ml of water Autoclave as above and run exactly as unknowns
7 Set blank at 100% T at 5 0 mμ

Calculation

1 (Total creatinine − preformed creatinine) = Creatine (as creatinine)
   To convert creatine expressed as creatinine into the amount of creatine itself multiply by 1 16
3 (Total creatinine − preformed creatinine) × 1 16 = Creatine

Example

1 Total creatine = 1 1 0 gm per liter urine
2 Preformed creatinine = 0 9 0 gm per liter
3 (1 1 0 − 0 9 0) 1 16 = 0 3 gm of creatine per liter urine

Precautions

1 The precautions are the same as for creatinine with one important addition the hydrolysis of creatine may be affected markedly by temperature and acidity Hence conditions during hydrolysis must be controlled rigidly

10 Urea Nitrogen

A. UREA NITROGEN IN BLOOD AND SERUM

Reference

Gentzkow C J and Masen J M An Accurate Method for the Determination of Blood Urea Nitrogen by Direct Nesslerization J Biol Chem 143 531 544 (Feb) 1942

Principle

The enzyme urease hydrolyzes urea into ammonium carbonate quantitatively at ordinary temperatures

Apparatus

1 Coleman Jr Spectrophotometer Model #6
2 Cuvettes 19 × 150 mm
3 15 ml round bottom centrifuge tubes
4 Water bath set at 55 C
5 Electric centrifuge
6 Syringe pipettes exactly 0 2 ml and 0 3 ml

Reagents

1 Urease 10% solution Mix 4 gm of urease (Squibbs Powder) 27 ml of distilled water and 13 ml of 95% ethyl alcohol This solution keeps only for one workin day
2 Sodium tungstate a 10% solution in water
3 Sulfuric acid 2/3 N Exactly 6 6 ml concentrated sulfuric acid (H_2SO_4) is made up to 360 ml with distilled water
4 Dilute Nessler s reagent Pla e 500 ml of 10% NaOH solution into a liter flask add 150 ml of Nessler s (for NPN) and dilute to 1000 ml with water Mix and use on same day
5 Standard solution of nitrogen Exactly 0 236 gm of dry ammonium sulfate is diluted to exactly one liter with distilled water Store in refrigerator 1 0 ml = 0 05 mg of nitrogen
6 Permutit prepared according to Fol n (Folin O and Bell R D Applications of a New Reagent for the Separation of Ammonia J Biol Chem 29 329 March 1917) Permutit is an insoluble sodium aluminum silicate (zeolite) that is used to absorb ammonia from solutions It is best prepared (freeing the ammonia) by washing with 10% NaOH and then with 2% acetic acid Decant wash with distilled water and dry in the air without heat The recommended powder to use is 60 mesh

Procedure

1 Add exactly ? 0 ml distilled water to a 15 ml centrifuge tube
2 Add exactly 0 ml serum with a syringe pipette
3 Add exactly 0 2 ml urease solution and incubate at 5 C for 15 minutes
4 Add exactly 0 3 ml of sodium tungstate solution
5 Add exactly 0 3 ml of 2/3 N sulfuric acid and allow to flocculate
6 Add exactly ml of distilled water Mix by inversion Centrifuge until clear

7 Into a cuvette add exactly 5 ml of supernatant fluid and 5 ml of water
8 Prepare a blank by adding ^ ml of dilute Nessler s solution to 10 ml of water
9 Set blank at 100% T at a wave length of 500 mμ
10 Add 2 ml of Nessler s to the unknowns and *read immediately*
11 Prepare a standard curve using a range of 0 1 mg to 0 07 mg

Calculation

$$\text{mg urea N/100 ml serum} = \frac{(\text{mg N in cuvette}) \times 10 \times 100}{(\text{ml aliquot}) \times (\text{ml serum})}$$

Example

A *reading of 65% T* was obtained equal to 0 0 mg N *in* the cuvette Of serum 0 ° ml was used and a 5 ml aliquot of the supernatant fluid

$$\text{mg urea N/100 ml serum} = \frac{(0\ 0\ \ ) \times 10\quad 100}{(5) \times (0\ \ )} = 0\ 0 \text{ mg urea N}$$

Precautions

1 The samples must be read immediately as they cloud up n 1 to minutes Incubation time and temperature are important
3 The urease solution is unstable and must be replaced daily

B UREA NITROGEN IN URINE

Procedure

1 To a 50 ml flask add approximately 5 ml of urine
2 Add approximately gm of permutit Stoppe and shake for 5 minute (The permutit absorbs the ammonia from the u ne)
3 Allow to settle and decant off clear supernatant fluid
4 Dilute exactly 1 ml of ammonia free urine to exactly 50 ml with water
5 Pipette exactly 1 0 ml of d luted urine into a 15 ml centrifuge tube
6 Ad 1 exactly 1 0 ml distilled water
7 Add exactly 0 ml of urease solution and incubate for 15 minutes at 55 C
8 Add 0 4 ml of sod um tungstate solution
9 Add 0 4 ml of /3 N sulfuric acid and dilute to 10 ml (ml water)
10 Allow to flocculate and centrifuge until clear
11 Use 0 ml of supernatant fluid in a cuvette
1 Add 8 0 ml distilled water
13 Add 2 0 ml dilute Nessler s reagent and read immediately at 500 mμ
14 Set blank consisting of 10 ml of water and ml of Nes ler solution at 100% T
15 Use same standard curve as for se um

Calculation

$$\text{gm urea N/liter urine} = \frac{(\text{mg N in cuvette}) \times (\text{ml d lution}) \times (1000)}{(1000) \times (\text{ml al quot}) \times (\text{ml urine})}$$

Example

A read ng of 63 0 T was equivalent to 0 0_1 mg N in the cuvette Dilution was One ml of urine was d luted to 50 of this 1 ml was diluted to 10 An aliquot of 2 0 ml was taken fo analysis

$$\text{gm urea N/liter urine} = \frac{(0\ 0\ 1) \times (50) \times (10) \times (1000)}{(1000) \times (\ \ 0) \times (1)} = 5\ ^\circ 5 \text{ gm N}$$

Precautions

Same as for urea nitrogen in s rum and blood

Precautions

1 The precautions are the same as for creatinine with one important addition the hydrolysis of creatine may be affected markedly by temperature and acidity Hence conditions during hydrolysis must be controlled rigidly

10 Urea Nitrogen

A. UREA NITROGEN IN BLOOD AND SERUM

Reference

Gentzkow C J and Masen J M An Accurate Method for the Determination of Blood Urea Nitrogen by Direct Nesslerization J Biol Chem 143 531 544 (Feb) 1942

Principle

The enzyme urease hydrolyzes urea into ammonium carbonate quantitatively at *ordinary temperatures*

Apparatus

1 Coleman Jr Spectrophotometer Model #6
2 Cuvettes 19 × 150 mm
3 15 ml round bottom centrifuge tubes
4 Water bath set at 55 C
5 Electric centrifuge
6 Syringe pipettes exactly 0 2 ml and 0 3 ml

Reagents

1 *Urease 10% solution Mix 4 gm of urease (Squibbs Powder) 27 ml of* distilled water and 13 ml of 95% ethyl alcohol This solution keeps only for one working day
2 Sodium tungstate a 10% solution in water
3 Sulfuric acid 2/3 N Exactly 6 6 ml concentrated sulfuric acid (H_2SO_4) is made up to 60 ml with distilled water
4 *Dilute Nessler s reagent* Place 500 ml of 10% NaOH solution into a liter flask add 150 ml of Nessler s (for NPN) and dilute to 1000 ml with water Mix and use on same day
5 Standard solution of nitrogen Exactly 0 236 gm of dry ammonium sulfate is diluted to exactly one liter with distilled water Store in refrigerator 1 0 ml = 0 05 mg of nitrogen
6 Permutit prepared according to Folin (Folin O and Bell R D Applications of a New Reagent for the Separation of Ammonia J Biol Chem. 29 3 9 March 1917) Permutit is an insoluble sodium aluminum silicate (zeolite) that is used to absorb ammonia from solutions *It is best prepared (freeing* the ammonia) by washing with 10% NaOH and then with 2% acetic acid Decant wash with distilled water and dry in the air without heat The recommended powder to use is 60 mesh

Procedure

1 Add exactly 2 0 ml distilled water to a 15 ml centrifuge tube
2 Add exactly 0 ? ml serum with a syringe pipette
3 *Add exactly 0 2 ml urease solution* and incubate at 55 C for 15 minutes
4 Add exactly 0 3 ml of sodium tungstate solution
5 Add exactly 0 3 ml of 2/3 N sulfuric acid and allow to flocculate
6 Add exactly 7 ml of distilled water Mix by inversion Centrifuge until clear

Into a cuvette add exactly 5 ml of supernatant fluid and 5 ml of water

8 Prepare a blank by adding ‿ ml of dilute Nessler s solution to 10 ml of water
9 Set blank at 100% T at a wave length of 500 mμ
10 Add ‿ ml of Nessler s to the unknowns and read immediately
11 Prepare a standard curve using a range of 0 1 mg to 0 07 mg

Calculation

$$\text{mg urea N/100 ml serum} = \frac{(\text{mg N in cuvette}) \times 10 \times 100}{(\text{ml aliquot}) \times (\text{ml serum})}$$

Example

A read ng of 65% T was obtained equal to 0 0 mg N in the cuvette Of serum 0.2 ml was used and a 5 ml aliquot of the supernatant fluid

$$\text{mg urea N/100 ml serum} = \frac{(0\,0\ \ ) \times 10 \times 100}{(5) \times (0\ \ )} = 0\,0 \text{ mg urea N}$$

Precautions

1 The samples must be read immediately as they cloud up in 1 to 2 minutes Incubation time and temperature are important
3 The urease solution is unstable and must be replaced da ly

B UREA NITROGEN IN URINE

Procedure

1 To a 50 ml flask add approximately 5 ml of urine
2 Add approximately gm of permut t Stopper and shake for 5 minutes (The permutit absorbs the ammonia from the urine)
3 Allow to settle and decant off clea supernatant flu d
4 Dilute exactly 1 ml of ammonia free urine to exactly 50 ml with water
5 Pipette exactly 1 0 ml of diluted urine nto a 15 ml centrifuge tube
6 Add exactly 1 0 ml d stilled water
7 Add exactly 0 2 ml of urease solution and incubate fo 15 minutes at 55 C
8 Add 0 4 ml of sod um tungstate solution
9 Add 0 4 ml of ²/3 N sulfuric ac d and dilute to 10 ml (7 ml water)
10 Allow to flocculate and cent fuge until clear
11 Use 0 ml of supernatant fluid in a cuvette
1 Add 8 0 ml d st lled wate
13 Add 0 ml dilute Nessler s reagent and read immediately at 500 mμ
14 Set blank consisting of 10 ml of water and ml of Ne sler s solution at 100% T
15 Use same standard curve as for serum

Calculation

$$\text{gm urea N/l ter urine} = \frac{(\text{mg N in c ivette}) \times (\text{ml d lut on}) \times (1000)}{(1000) \times (\text{ml ali quot}) \times (\text{ml urine})}$$

Example

A reading of 63 0% T was equivalent to 0 0 1 mg N in the cuvette Dilution was One ml of urine was d luted to 50 of this 1 ml was diluted to 10 An al quot of ² 0 ml was taken for analysis

$$\text{gm urea N/liter urine} = \frac{(0\,0\,1) \times (50) \times (10) \times (1000)}{(1000) \times (\ \ 0) \times (1)} = 5\ \ \text{gm N}$$

Precautions

Same as for urea n t ogen in s um and blood

11 Amino Acid Nitrogen in Urine

Reference

Folin O A Colorimetric Determination of the Amino Acid Nitrogen in Normal Urine J Biol Chem. 51 393 394 (April) 1922

Principle

The 1 to 2 percent of the total nitrogen which is combined as amino acids in the urine is estimated colorimetrically

Apparatus

1 Cuvettes 25 x 105 mm
  Coleman Jr Spectrophotometer Model #6
3 Tubes graduated to 25 ml
4 Dark room or equivalent
5 Accurately calibrated 5 ml syringe pipette

Reagents

1 Permutit
2 1 25% sodium carbonate in water
3 0 1 N hydrochloric acid (HCl)
4 Sodium beta napthoquinone-4 sulfonate 0 5% in water
5 Phenolphthalein indicator
6 Sodium acetate 25% in a 25% aqueous solution of acetic acid
  Sodium thiosulfate 4% in water
8 Standard solution Amino acetic acid 0 536 gm plus 2 0 gm sodium benzoate up to 100 ml 0 1 N HCl (0 1 mg of amino acid nitrogen per ml)

Procedure

1 Dilute exactly 5 0 ml of urine to 25 ml add 1 0 gm of permutit and shake (This step removes free ammonia)
2 Decant into another 3 gm of permutit Shake and decant again
3 Pipette 5 0 ml of ammonia free diluted urine into a 25 ml cuvette
4 Add 1 0 ml of 0 1 N HCl
5 Add 1 0 ml of 1 25% sodium carbonate and dilute to 10 ml
6 Then add 5 ml of sodium b napthoquinone-4 sulfonate solution and mix.
7 Stand overnight in dark and make acid to phenolphthalein
8 Add 5 0 ml of sodium thiosulfate and dilute to 25 ml
9 Set blank at 100% T and read at 480 mμ
10 Set up standard curve using 100 to 400 mg amino acid nitrogen

Calculation

$$\text{mg amino N/liter urine} = \frac{(\text{mg amino N in cuvette}) \times (\text{dilution}) \times 1000}{(\text{ml aliquot in cuvette}) \times (\text{ml urine in original sample})}$$

Example

A reading of 29 0% T was equivalent to 0.20 mg amino nitrogen in cuvette Of urine 5 ml samples were diluted to 25 ml of which 5 ml was analyzed.

$$\text{mg amino N/liter urine} = \frac{(0\ 20) \times (25) \times (1000)}{(5) \times (5)} = 200 \text{ mg amino nitrogen}$$

Precautions

1 Depending on the intensity of color developed at Step 8 more or less diluted urine may be required at Step 3

12 Uric Acid

A. URIC ACID IN SERUM

Reference

Benedict S R and Franke E A Method for the Direct Determination of Uric Acid in Urine J Biol Chem 52 38 391 (June) 19 2

Principle

Ur c acid reduces phosphotungstic acid and cyan de with the formation of a blue color which is measured color metrically

Apparatus

1 Coleman Jr Spectrophotometer Model #6
2 Cuvettes 19 × 150 mm
3 A quantity of 15 ml round bottom centrifuge tubes
4 Electric centrifuge
5 Water bath set at 5 C
6 Calibrated syringe pipette 0 5 ml
7 Burette

Reagents

1 Sod um tungstate (Na_2WO H O) 10% solution in water
2 Sulfur c acid (H SO) /3 N
   6 6 ml concentrated sulfur c acid made up to 360 ml with distilled water
3 L thiu n chlor de (LiCl)
   3 gm LiCl and 1 ml hydrochlo c acid (conc) made up to 1 liter with water
4 S lver nitrate (AgNO)
   11 6 gm silver nitrate per l ter dist lled water
5 Sodium cyanide (NaCN) a 10% solution in water freshly made *POISON*
6 Color reagent Arsenophosphotungstic acid *POISON*
   Stock. D ssolve 100 gm sod um tungstate (Na_2WO H O) in about 600 ml of water in a l ter flask Add 50 gm pure arsenic pentox de followed by 5 ml of syrupy phosphoric acid (85%) and 0 ml concentrat d hydrochloric acid Bring to a boil and add 10 ml of brom ne water (The stock solution is 0 ml water saturated with bromine) Boil until color d sappears and continue to boil until all traces of bromine are driven off Dilute to 1000 ml This color reagent keeps indefinitely
   For use dilute stock color reagent with equal port ons of water before use
7 Benedict Hitchcock Stock Standard Solut on
   Dissolve 9 gm of pure crystall zed d sodium phosphate (Na_2HPO 12H O) and 1 gm monobas c sodium pho phate (NaH PO H O) in about 50 ml hot water Filter f not clear Make up to 500 ml w th hot water and pour this solution on exactly 00 mg of pure uric acid suspended in a few ml of water in a liter volumetric flask Shake gently unt l uric acid is in solution Cool and add exactly 1 4 ml of glacial acet c acid dilute to 1 l ter and mix well Add 5 ml chlo oform to prevent bacterial g owth Keep in refrigerator Good for two months or more 1 ml = 0 . mg u c a id
8 D lut uric ac d standard made by d lut ng stock ur c ac d sta da d 1 0 so that 1 ml conta ns 0 01 mg uric acid

Procedure (Se um)

1 Into a 15 ml round bottom centrifuge tube p pette exactly 0 ml eru from an accurately cal brated syringe pipette
2 Add exactly 8 ml water
3 Add exactly 0 5 ml sodium tungstate solution (Na_2WO H O)

4 Then add exactly 0 5 ml sulfuric acid (H_2SO)
5 Stopper shake vigorously for approximately 15 seconds and centrifuge for 5 minutes
6 Transfer exactly 5 ml of the above filtrate into another 15 ml centrifuge tube
7 Add exactly 2 5 ml lithium chloride solution (LiCl) and mix by inversion
8 Then add exactly 2 5 ml silver nitrate solution (Steps 7 8 and 9 are said to remove non uric acid interfering substances)
9 Stopper mix and centrifuge for 5 10 minutes Disregard cloudiness of solution
10 Drain quantitatively into a cuvette
11 Add exactly 0 5 ml of color reagent *POISON*
12 Add 1 ml fresh NaCN Use *burette or syringe pipette* for both steps 11 and 12
13 Let stand for 15 minutes at 25 C then read in a spectrophotometer with wave length at 540 mμ The instrument should be set at 100% T against a blank of 10 ml water 0 5 ml color reagent and 1 0 ml cyanide

Calculation

1 Make up a standard curve using from 5 mcg to 35 mcg of uri acid per sample at intervals of 5 gamma
2 mg uric acid/100 ml serum =

$$(\text{mg uric acid in cuvette}) \times \frac{(\text{dilution})}{(\text{ml aliquot})} \times \frac{100}{(\text{ml serum})}$$

Example

A reading of 78% T was equivalent to 0 008 mg uric acid in the cuvette Of serum 0 5 ml was diluted to 10 ml of which 5 were taken for analysis

$$\text{mg uric acid/100 ml serum} = (0\ 008) \times \frac{10}{5} \times \frac{100}{0\ 5} = 3\ \text{mg}$$

Precautions

1 Use a burette for both the cyanide and color reagent
2 The cyanide should be made up every two weeks and kept in the refrigerator
3 Time and temperature are critical

B URIC ACID IN URINE

Principle Apparatus Reagents

Same as for uric acid in serum

Procedure

1 Dilute the urine routinely 1 to 20 ml being satisfactory Occasionally 1 to 50 may be necessary
2 Pipette exactly 1 ml of the dilute urine into a cuvette
3 Add exactly 9 ml of water
4 Proceed as for *Serum* steps 11 12 and 13

Calculation

1 Use the same standard curve as for *Serum*
2 mg uric acid/liter urine =

$$(\text{mg uric acid in cuvette}) \times \frac{(\text{dilution})}{(\text{ml aliquot})} \times \frac{(1000)}{(\text{ml urine used})}$$

Example

A reading of 67% T was equivalent to 0 012 mg uric acid in the cuvette One ml of urine was diluted to 20 of which one ml was used for analysis

$$\text{mg uric acid/l urine} = 0\ 012 \times \frac{20}{1} \times \frac{1000}{1} = 240\ \text{mg uric acid/l}$$

Precautions

Same as for *Serum*

13 Uric Acid (Uricase Method)

A. URIC ACID IN URINE

Reference

Buchanan O H Block W D and Christman A A The Metabolism of the Methylated Purines I The Enzymatic Determination of Urinary Uric Acid J Biol Chem 157 181 201 (Jan) 1945

Principle

The enzyme uricase when incubated with urine specifically destroys all the uric acid. Therefore by measuring the color intensity of a specimen before and after *incubation one can calculate the true uric acid in the urine*

Apparatus

1 Coleman Jr Spectrophotometer Model No 6
2 Cuvettes 19 × 150 mm
3 A quantity of 15 ml round bottom centrifuge tubes
4 An electric centrifuge
5 A water bath set at 5 C
6 A water bath set at 45 C
7 Calibrated syringe pipettes 1 ml ( ) and 2 5 ml
8 Calibrated ml burettes ( )
9 A good food chopper or Waring Blendor
10 A refrigerator set at 4 C
11 *A quantity of 50 ml volumetric flasks*
12 A quantity of filter paper
13 An interval timer

Reagents

1 Color reagent arsenophosphotungstic acid (for preparation see uric acid in serum) *POISON*
2 Urea cyanide solution Dissolve [illegible] gm of sodium cyanide and 50 gm of anhydrous sodium carbonate in 400 ml of water Cool add 5 gm of urea and dilute *to 500 ml with water* This solution is stable for several months even though a slight precipitate will settle out on standing *POISON*
3 Uric acid standard solution 0 01 per cent This solution is made up in a 0 15 per cent solution of lithium carbonate (1 5 gm dissolved in 1000 ml of distilled water)
4 Sodium carbonate 0 1 [illegible] solution Dissolve 1 [illegible] gm Na_2CO_3 in 1000 *ml of water*
5 Toluene C P (reagent grade)
6 Sodium tungstate a 10% solution in water
7 Sulfuric acid 2/3 N
8 Acetone C P (reagent grade)
9 Benzene C P (reagent grade)
10 Sodium hydroxide an 0 1 N solution
11 Borate buffer (pH 9) Dissolve 1 4 gm of boric acid in 1000 ml of 0 1 N sodium hydroxide
12 *Thymol blue indicator 0 04% solution*
13 Uricase preparation of (M B Blauch and F C Koch A New Method for the Determination of Uric Acid in Blood With Uricase J Biol Chem 130 443 454 Oct 1939)
   a One pound of fresh beef kidney is mechanically *defatted and then ground* finely in a food chopper or Waring Blendor
   b Dehydrate with 4 washings of acetone (500 ml each) over a period of 48 hours at 4 C 12 hours being allowed for each extraction

c Dry the tissue quickly with the aid of a fan at room temperature
d. Treat the dry material twice (at room temperature) with 500 ml of benzene the first treatment for 6 hours followed by filtering and the second for 1? hours
e The material is dried at room temperature and then ground to 60 mesh.
f Store at 4 C

14 *Measurement of uricolytic activity*
a To 10 ml of a standard uric acid solution (0 01% in 0 15% lithium carbonate) add 1 ml of 0 125% sodium carbonate
b Add 1 drop of toluene and increasing amounts of the kidney pulp
c Incubate for two hours at 45 C with occasional stirring
d Add 30 ml of distilled water
e *Add 1 ml of a 10% sodium tungstate solution*
f Add 2 ml of 2/3 N sulfuric acid
g Shake and filter
h Then determine the uric acid content in the clear filtrate
i The amount of uricase to be used per analysis should be determined as the point where all the uric acid is destroyed 250 mg of the powder should destroy 3 4 mg of uric acid after incubation

Procedure Total Color

1 Pipette exactly 1 ml of urine (using a syringe pipette) into a 50 ml flask, dilute to the mark with distilled water and mix
2 To another 50 ml flask transfer exactly 10 ml of the diluted urine and add 15 ml of water
3 To a second flask add only 25 ml of water This sample will be the blank
4 To each flask add 2 5 ml of urea cyanide solution using a syringe pipette
5 Then add with a syringe pipette 1 ml of arsenophosphotungstic acid
6 Immediately after the addition of the color reagent dilute to the 50 ml mark and mix thoroughly
7 Allow to stand at room temperature for exactly 30 minutes and read the samples at 690 mμ, setting the blank at 100% T

Procedure Residual Color

1 Pipette exactly 5 ml of undiluted urine into a small flask add 10 ml of water and a few drops of 0 04 per cent thymol blue solution as indicator
2 Titrate with 0 1 N sodium hydroxide to a definite blue tint and record the amount of alkali required Discard the mixture
3 *Into a 50 ml flask pipette exactly 5 ml of undiluted urine*
4 Add the predetermined amount of alkali (without indicator)
5 Add 250 mg of uricase powder
6 Add 5 ml of borate buffer and wash down the sides of the flask with 10 ml of water
7 Place in a water bath at 45 C for 2 hours
8 Then add 1 ml of sodium tungstate solution and 1 5 ml of 2/3 N sulfuric acid.
9 Dilute to 50 ml mark with water mix well and pour into a dry filter
10 Pipette exactly 10 ml of the filtrate into a 50 ml volumetric flask and *add 15* ml of water
11 Continue with the addition of the color reagents and measure the color in the *same way as* described above for the total color
12 The same blank may be used or a 5 ml portion may be treated with uricase and all the other reagents

Calculation

1 (Total color) minus (Residual color) = true uric acid content
2 mg true uric acid per 100 ml urine =

$$\left[\text{mg (total color) uric acid in cuvette} \times \frac{\text{dilution}}{\text{ml aliquot}} \times \frac{100}{\text{ml urine used}}\right]$$

minus

$$\left[\text{mg (residual color) uric acid in cuvette} \times \frac{\text{dilution}}{\text{ml aliquot}} \times \frac{100}{\text{ml urine used}}\right]$$

Example

1 The reading for the total true color was equivalent to 0 006 mg uric acid in the cuvette One ml of urine was diluted to 50 ml of which 10 ml was used and diluted to 50 ml 10 ml was read in the cuvette
2 The reading for the residual color was equivalent to 0 004 mg uric acid 5 ml of urine was diluted to 50 ml of which 10 ml was used in the final sample and diluted to 50 ml 10 ml was read in the cuvette

$$\text{Total color mg/100 ml} = 0\ 006 \times \frac{50}{10} \times 10 \times \frac{100}{1} = 30 \text{ mg/100 ml}$$

$$\text{Residual color mg/100 ml} = 0\ 004 \times \frac{50}{10} \times 10 \quad \frac{100}{5} = 4 \text{ mg}$$

Therefore True uric acid mg/100 ml = 30 − 4 = 6 mg

Precautions

1 Use a burette or a syringe pipette for the sodium cyanide and urea cyanide They are strong poisons
2 Be sure to measure the uricolytic activity of each new batch of uricase
3 Uricase is available commercially However for most laboratories the price is prohibitive

14 Paper Chromatography

This technique has afforded a new tool whereby many biological factors may be studied One may investigate the amino acids in plant and animal tissue with a minimum amount of preparation and destruction Only small quantities are required to demonstrate the presence of amino acids Nor is its use confined solely to the estimation of amino acids Its simplicity has permitted its ready adaptation to the analysis of such substances as sugars and organic acids With proper development it holds more promise to the chemistry of proteins carbohydrates organic acids and to the isotopic derivatives thereof than any other technique in the last decade

During the past few years filter paper chromatography has been applied to many different categories of compounds The bibliography below is by no means complete but will serve to introduce the prospective user to some of the various phases of this versatile method

Methodology

Brimley R C Quantitative Paper Chromatography Nature 163 215 16 (Feb) 1949
Bull H B Advances in Protein Chemistry by M L Anson and J T Edsall New York City 1947 Academic Press vol 3 p 97

Bull H B Hahn J W and Baptist, V H Filter Paper Chromatography J Am. Chem. Soc 71 550 553 (Feb) 1949
Chargaff E Levine C and Green C Techniques for the Demonstration by Chromatography of Nitrogenous Lipide Constituents Sulfur Containing Amino Acids and Reducing Sugars J Biol Chem 175 67 71 (Aug) 1948
Consden R and Gorden A H Effects of Salt on Partition Chromatograms, Nature 16? 180 181 (July) 1948
Dent C E Detection of Amino acids in Urine and Other Fluids Lancet 2 18 637 (Nov) 1946
Dunabin J E Mason H Seyfang A P and Woodman F J Spectrographic Examination of Chromatographic Columns Nature 164 916 (Nov) 1949
Fink R M Dent C E and Fink K Application of Filter Paper Partition Chromatography to Radioactive Tracer Studies Nature 160 801 803 (Dec) 1947
Fink R M and Fink K Radiocarbon and Filter Paper Partition Chromatography Science 107 253 254 (March) 1948
Fisher, R B Parsons D S and Morrison G A Quantitative Paper Chromatography Nature 161 764 765 (May) 1948
Fisher, R B Parsons D S and Holmes R Quantitative Paper Chromatography Nature 164 183 (July) 1949
Keston A S Udenfriend S and Levy M Paper Chromatography Applied to the Isotopic Derivative Method of Analysis J Am Chem. Soc 69 3151 3152 (Dec) 1947
La Cour L F and Drew R Partition Chromatography and Living Cells Nature 159 4035 307 (March) 1947
Longnecker W H *Glass Trough for Filter Paper Partition Chromatography Science 107* 23 24 (Jan) 1948
Lugg J W H and Overell B T Partition Chromatography of Organic Acids on a Paper Sheet Support Nature 160 4055 87 88 (July) 1947
Martin A J P Partition Chromatography Ann N Y Acad Sci 49 249 264 (Feb) 1948
Meinhard J E and Hall N F Surface Chromatography Anal Chem 21 185 188 (Jan) 1949
Meinhard J E and Hall N F Surface Chromatography Anal Chem 22 (2) 344 351 (Feb) 1950
Miettinen J K and Virtanen A I A New Technique in Paper Chromatography Acta Chem Scandinav 3 459 464 No 5 1949
Nicholson D E Modified Technique for the Development of Paper Chromatograms Nature 163 954 (June) 1949
Phillips D M P Use of Ultra Violet Fluorescence in Paper Chromatography Nature 161 53 (Jan) 1948
Pollard F H McOmie J F W and Elbeih I I M Inorganic Paper Chromatography and Detection of Cations by Fluorescence Nature 163 216 ?0 (Feb) 1949
Rutter L A Modified Technique in Filter Paper Chromatography, Nature 161 435 436 (March) 1948
Schroeder W A Some Experiments in Systematic Quantitative Chromatography Ann N Y Acad Sci 49 04 217 (Feb) 1948
Williams R J and Kirby H Paper Chromatography Using Capillary Ascent, Science 107 481 483 (May) 1948
Winsten W A A Simplified Apparatus for One Dimensional Paper Partition Chromatography Science 107 60? (June) 1948
Wynn V Peptide like Contaminant of Filter Paper Nature 164 445 1949
Zechmeister L History Scope and Methods of Chromatography Ann. N Y Acad. Sci. 49 145 160 (Feb) 1948

Amino Acids

Agren G A Note on the Amino Acid Content of Bence Jones Protein Acta Chem Scand 3 (3) 301 30? 1949
Agren G and Nilsson, T Paper Chromatographic Analysis of Amino Acids and Other Ninhydrin Reacting Substances in Deproteinized Human Plasma Acta Chem Scandinav 3 5?5 538 No ? 1949
Ames S R and Risley H A Aminoaciduria in Progressive Muscular Dystrophy Fed. Proc 7 1 142 (March) 1948
Awapara J Application of Paper Chromatography to the Estimation of Some Free Amino Acids in Tissues of the Rat J Biol Chem 178 113 116 (March) 1949
Berry H K and Cain L Biochemical Individuality IV Paper Chromatographic Technique for Determining Excretion of Amino Acids in the Presence of Interfering Substances Arch Biochem 24 (1) 1 9 189 (Nov) 1949
Block R J Quantitative Estimation of Amino Acids on Paper Chromatograms Science 108 608 609 (Nov) 1948

Block R J Quantitative Paper Chromatography A Simplified Procedure Proc Soc Exper Biol & Med. 72 (?) 337 341 (Nov) 1949

Consden P Gordon A H and Martin A J Qualitative Analysis of Proteins A Partition Chromatographic Method Using Paper Biochem J 38 224 23 1944

Consden R Gordon A H and Martin A J The Identification of Amino Acids Derived From Cystine in Chemically Modified Wool, Biochem J 40 580 58? 1946

Consden R Gordon A H and Martin A J Gramicidin S The Sequence of Amino Acid Residues Biochem. J 41 596 60? 1947

Dent C E Partition Chromatography on Paper as Applied to the Investigation of Amino Acids and Peptides in Normal and Pathological Urine Biochem. J (Proc.) 40 (4) xliv xlv 1946

Dent C E Partition Chromatography on Paper A Qualitative Method for Identifying Amino Acids in Urine and Its Application to the Fanconi Syndrome Trans of Fourteenth Meeting Conference on Metabolic Aspects of Convalescence New York (Nov) 1946

Dent C E The Amino Aciduria in Fanconi Syndrome A Study Making Extensive Use of Techniques Based on Paper Partition Chromatography Biochem J 41 (?) 240 253 194

Dent C E Methionine Metabolism and Alpha Aminobutyric Acid Science 105 33 336 (March) 1947

Dent C E Stepka W and Steward F C Detection of Free Amino Acids of Plant Cells by Partition Chromatography Nature 160 40 2 68? (Nov) 1947

Goldberg H J V Gilda J E and Tishkoff G H Paper Partition Chromatography Free Amino Acids in Saliva J Dent Research 27 493-496 (Aug) 1948

Gordon A H Martin A J and Synge R L Partition Chromatography of Free Amino Acids and Peptides Biochem J 37 Proc xiii 1943

Keston A S Udenfriend S and Levy M Quantitative Analysis of Protein Hydrolysates on Paper Chromatograms by Means of the Isotopic Derivative Method Fed Proc 107 1 164 (March) 1948

Landua A J and Awapara J Use of Modified Ninhydrin Reagent in Quantitative Determination of Amino Acids by Paper Chromatography Science 109 385 (April) 1949

Martin A J P and Mittelmann R Quantitative Microanalysis of Amino Acid Mixtures on Paper Partition Chromatograms Biochem J 43 353 58 1948

Moore S and Stein W H Partition Chromatography of Amino Acids on Starch Ann N Y Acad Sc 49 ?5 8 (Feb) 1948

Moore S and Stein W H Photometric Ninhydrin Method for Use in the Chromatography of Amino Acids J Biol Chem 176 367 388 (Oct) 1948

Moore S and Stein W H Chromatography of Amino Acids on Starch Columns Solvent Mixtures for the Fractionation of Protein Hydrolysates J Biol Chem 178 53 78 (March) 1949

Naftalin L Quantitative Chromatographic Estimation of α Amino Acids Nature 161 763 764 (May) 1948

Polson A Mosley V M and Wyckoff R G W The Quantitative Chromatography of Silk Hydrolysat S n e 105 603 604 (June) 194

Pope C G and Stevens M F The Determination of Amino Nitrogen Using a Copper Method Biochem J 33 10 0 10 9

Rockland L B and Dunn M S Quantitative Determination of Amino Acids on Filter Paper Chromatograms by Direct Photometry J Am Chem Soc 71 (1) 41 1-41 (Dec) 1949

Sanger F The Free Amino Groups of Insulin Biochem J 39 50 515 194

Stein W H and Moore S Chromatography of Amino Acids on Starch Columns Separation of Phenylalanine Leucine Isoleucine Methionine Tyrosine and Valine J Biol Chem 176 337 365 (Oct) 1948

de Verdier C H and Agren G Paper Chromatographic Analysis of Amino Acids and Peptides in Tissue Extracts and Enzyme Hydrolyzed Proteins Acta Chem Scandinav 2 783 79? 1948

Winegard H M and Toennies G Detection of Sulfur Containing Amino Acids on Paper Chromatograms Science 108 506 507 (Nov) 1948

Woiwod A J Micro Estimation of Amino Nitrogen and Its Application to Paper Partition Chromatography Nature 161 169 (Jan) 1948

Woiwod A J Method for the Estimation of Micro Amounts of Amino Nitrogen and Its Application to Paper Partition Chromatography Biochem J 45 412 417 1949

Young N F, and Homburger F The Application of Paper Chromatography to the Study of Amino aciduria in Patients With Liver Disease Fed. Proc ? 1 ?1 (March) 1949

Carbohydrates

Brown F Hirst E L Hough L Jones J K N and Wadman H. Separation and Identification of Methylated Sugars on the Paper Chromatogram, Nature 161 720 (May) 1948

Flood A E Hirst E L and Jones J K N Quantitative Analyses of Mixtures of Sugars by the Method of Partition Chromatography Part I. Standardization of Procedure J Chem Soc 128 1679 1683 1948

Flood A E Hirst E L and Jones J K Quantitative Estimation of Mixtures of Sugars by the Paper Chromatogram Method Nature 160 4055 86 (July) 1947

Horrocks R H and Manning G B Partition Chromatography on Paper Identification of Reducing Substances in Urine Lancet 256 1042 1045 (June) 1949

Hough L Jones J K N and Wadman W H Application of Paper Partition Chromatography to the Separation of the Sugars and Their Methylated Derivatives on a Column of Powdered Cellulose Nature 162 448 (Sept) 1948

Partridge S M with a *note by* Westall R G *Filter Paper Partition Chromatography* of Sugars 1 General Description and Application to the Qualitative Analysis of *Sugars in Apple Juice Egg White and Foetal Blood of Sheep* Biochem J 42 238 250 No 2 1948

Partridge S M *Application of Paper Partition Chromatogram to the Qualitative Analy*sis of Reducing Sugars Nature 158 4008 270 (Aug) 1946

Miscellaneous

Allsopp A Chromatographical Study of Meristematic Plant Tissue Nature 161 4100 833 835 (May) 1948

Ames S and Risley, H A *Determination of Creatine Creatinine* and Related Compounds in Urine by Means of Paper Chromatography Proc Soc Exper Biol & Med. 69 267 269 (Oct Dec) 1948

Bate Smith E C Paper Chromatography of Anthocyanins and Related Substances in Petal Extracts Nature 161 4100 835 838 (May) 1948

Bentley H R and Whitehead J K Use of Furan Derivatives in Paper Chromatography Nature 164 182 183 (July) 1949

Cavallini D Frontali N and Toschi G Determination of Keto Acids by Partition Chromatography on Filter Paper Nature 163 568 569 (April) 1949

Christ C L Burton C J and Botty M C Use of X Ray and Electron Diffraction as Methods of Analysis in Biochemical Chromatography, Science 108 91 93 (July) 1948

Crammer J L Paper Chromatography of Flavin Nucleotides Nature 161 4088 350 (March) 1948

Dingemanse E Huis in t Velt L G and de Laat B M Clinical Method for the Chromatographic Colorimetric Determination of Urinary 17 Keto Steroids J Clin Endocrinol 6 (8) 535 548 (Aug) 1946

Edman P Hammarsten E Low B and Reichard P Partition Chromatographic Separation of Adenine and Guanine J Biol Chem 178 395 398 (March) 1949

Ekman B Paper Chromatography of Primary Aromatic Amines Acta Chem. Scandinav 2 383 384 No 4 1948

Franklin A F and Quastel J H Paper Chromatography of Proteins and Enzymes Science 110 447 451 (Oct) 1949

Hais I M and Pecáková L Paper Partition Chromatography of Riboflavine Decomposition Products Nature 163 768 (May) 1949

Hess W C Chromatographic Separation of Cholesterol and Cholesterol Esters in Blood J Lab & Clin Med 32 1163 1168 (Aug) 1947

Holiday E R and Johnson E A Location of Paper Chromatogram Spots of Purine and Pyrimidine Derivatives in Ultra violet Light Nature 163 216 17 (Feb) 1949

Kluener R G A Paper Chromatographic Method for the Quantitative Estimation of Penicillin Entities J Bact 57 101 109 (Jan) 1949

Lacourt A Sommereyns G Degeyndt E Baruh J and Gillard J Quantitative Inorganic Paper Chromatography Sub Micro Separation and Determination of Aluminum Iron and Titanium Nature 163 999 1000 (June) 1949

Lederer M Chromatographic Separation of Antimony Anal Chim Acta 2 81 6 1948

Lederer M Paper Chromatography of the Noble Metals Nature 162 776 777 (Nov) 1948

Markham R and Smith J D *Chromatography of Nucleic Acid Derivatives* Nature 163 250 251 (Feb) 1949

Maw G A *The Detection of Creatine and Creatinine by Partition* Chromatography Biochem J 43 139 142 1948

Mitchell H K Gordon M and Haskins F A *Separation of Enzymes* on Filter Paper Chromatopile J Biol Chem 180 1071 1076 (Oct) 1949

Polson A New Amino Acids in Bacterial Hydrolysates Nature 161 4088 151 15 (March) 1948
Ramsey L L Separation of n Butyric and Isobutyric Acids by Part tion Chromatography J Assoc Offic Agr Chemists 31 164 1948
Ramsey L L and Patterson, W I Separation and Determination of the Straight Chain Saturated Fatty Acids C to C by Partition Chromatography J Assoc Offic Agr Chemists 31 139 150 1948
Tabone J Robert D and Troestler J Appl cation of Paper Strip Chromatography to the Analys s of Biological Arylamides Bull Soc Chim Biol 30 547 552 1948
Taurog A Tong W and Chaikoff I. L Fractionation of Thyroid Iodine by Means of Filter Paper Partition Chromatography Nature 164 181 18 (July) 1949
Tishkoff G H, Zaffaroni A and Tesluk H Purified Liver Extract Chem cal Nature as Determ ned by Paper Pa tition Chromatography J Biol Chem 175 857 86 (Sept) 1948
Tomarelli R M and Florey K Use of Papergrams in the Study of the Urinary Excretion of Radioactive Sulfur Compounds Science 107 630 631 (June) 1948
Urbach K. F and Giscafré L Identifi ation of H stamine in Blood by Paper Chromatography Proc Soc Exper Biol & Med 68 430 431 (May) 1948
Vischer E and Chargaff E The Separation and Characterization of Pu ines in Minute Amounts of Nucleic Acid Hydrolysates J Biol Chem 168 781 8 (May) 1947
Vischer E and Chargaff E The Separat on and Quantitat ve Estimation of Purines and Pyrimidines in Minute Amounts J Biol Chem 176 703 714 (Nov) 1948
White J W Jr Chromatograph c Separation of Al phatic 4 Dinitrophenylhydrazones Anal Chem 20 726 7 8 (Aug) 1948
White J W Jr and Dryden E C Separation of Aliphatic Alcohols by Chromatographic Adsorption of Their 3 5 Dinitrobenzoates Anal. Chem 20 853 855 (S pt) 1948
Winsten W A and E gen E Paper Chromatography of Vitamin B and Related Bacteriological Growth Factors J B ol Chem 181 (1) 109 1 0 (Nov) 1949
Woodruff H B and Foster J C Analysis of V tamin B and Vitam n B by Paper Strip Chromatography J Biol Chem 183 569 576 (Apr) 19 0
Zaffaroni A Burton R B and Keutmann E H The Application of Paper Partition Chromatography to Steroid Analysis J Biol Chem 177 109 116 (Jan) 1949
Zaffaroni A. Burton R B and Keutmann E H Adrenal Cortical Hormones Analysis by Paper Partition Chromatography and Occurrence in the Urine of Normal Persons Science 111 6 8 (Jan) 1950

15 Proteins and Nitrogenous Compounds Miscellaneous References

Alanine

Alexander B, and Seligman A M A Colorimetric Method for the Microdetermination of α Alanine in Blood J Biol Chem 159 9 19 (June) 1945
Wiss O Microdeterm nation of Alanine in Blood Helv Chim Acta 31 6 1948

Albumin and Globulin

Chow B F The Correlation Between the Albumin and Alpha Globulins Contents of Plasma J Clin Invest gation 26 (5) 883 886 (Sept) 1947
Cohn C and Wolfson W Q Stud es in Serum Prote ns I The Chemical Estimation of Albumin and of the Globul n Fractions in Serum J Lab & Clin Med 32 (10) 1 03 1 07 (Oct) 1947
Cohn C and Wolfson W Q Studies in Serum Proteins II A Rapid Clinical Method for the Accurate Determination of Albumin and Globul n in Serum or Plasma J Lab & Clin Med 33 (3) 367 370 (March) 1948
Isbister J The Estimat on of Serum Alb min by the Precipitation of Globul n with NaHSO Austral an J Exper B ol & Med 27 61 64 (Jan) 1949
K ngsley G P A Rapid Method for the Separat on of Serum Albumin and Globulin J Biol. Chem 133 731 735 (May) 1940
Petermann M L Young N F and Hogness K R A Compar son of the Howe and the Electrophoretic Methods for the Determination of Plasma Albumin J Biol Chem 169 (2) 379 387 (July) 1947

Allantoin

Larson H W A Colorimetr c Method for the Determination of Allantoin J B ol Chem. 94 (3) 727 7 8 (Jan) 193

Amino Acids

Moubasher R Estimat on of α Am no Ac ds in Pure Solut ons in Blood and in Urine With Peri Naphthindan 3 4 Tr one Hyd ate J Biol Chem 175 187 193 (Aug) 1948
Sahyun M Relat onship of Am no Acids to the Nutritive Value of Prote ns Am J Digestive Disease 16 (8) 301 05 (Aug) 1949

Amino Nitrogen

Danielson I S Amino Acid Nitrogen in Blood and Its Determination J Biol Chem 101 505 5°2 (July) 1933

Creatine

Fisher R B and Wilhelmi A E The Metabolism of Creatine I A Micro Method for the Determination of Creatine and Creatinine Biochem J 31 ( ) 1131 1135 1937
Rose W C Helmar O M and Chanutin A A Modified Method for the Estimation of Total Creatinine in Small Amounts of Tissues J Biol Chem 75 (ـ) 543 548 (Nov) 19°7
Addis T Barrett E and Menzies J T A Clinical Method for the Approximate Determination of Serum Creatinine Concentration J Clin Investigation 26 (5) 879 88 (Sept) 1947
Barclay J A and Kenney R A A Method for the Estimation of Creatinine Biochem J 41 (4) 586 589 1947

Cysteine

Nakamura K and Binkley F Colorimetric Estimation of Cysteine J Biol Chem. 173 407 410 (March) 1948

Histidine

Langley W D Urinary Histidine Determination of Histidine in Urine Normal and Pregnancy Urines J Biol Chem 137 °55 °66 (Jan) 1941

Non protein Nitrogen

Fee D A Cruger D and Collier, H B A Photometric Modification of the Hypobromite Method for Non protein Nitrogen J Lab & Clin Med 34 (6) 873 876 (June) 1949
Rappaport F and Lichhorn F Rapid Titrimetric Micromethod for the Determination of Non protein Nitrogen J Lab & Clin Med 32 (8) 1034 1036 1947
Wong S Y The Use of Persulphate in the Estimation of Nitrogen by Folin s Direct Nesslerization Method J Biol Chem. 55 431 436 (March) 19 3

Nucleic Acid

Steele R Sfortunato T and Ottolenghi L A Micromethod for the Determination of the Nucleic Acid J Biol Chem 177 ـ31 235 (Jan) 1949

Plasma Protein

Albanese A A Saur B and Irby V The Microcolorimetric Estimation of Plasma Protein J Lab & Clin Med 32 (2) ـ96 299 1947
Barbour H G and Hamilton W F The Falling Drop Method for Determining Specific Gravity J Biol Chem 69 (2) 6 5 640 (Aug) 19°6
Cohn E J Strong L E Hughes W L Jr Mulford D J Ashworth J N Melin M and Taylor H L Preparation and Properties of Serum and Plasma Proteins IV A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids J Am Chem Soc 68 459 475 (March) 1946
Hunter D Nesslerization Technic and Its Effect on Serum Protein Values Am J Clin Path 17 (8) 6ـ0 653 (Aug) 1947
Kagan B M A Simple Method for the Estimation of Total Protein Content of Plasma and Serum I A Falling Drop Method for the Determination of Specific Gravity II. The Estimation of Total Protein Content of Human Plasma and Serum by the Use of the Falling Drop Method J Clin Investigation 17 369 376 (July) 1938
Lowry O H and Hunter T H The Determination of Serum Protein Concentration With a Gradient Tube J Biol Chem 159 465 4،4 (July) 1945
Mulford D J Derivatives of Blood Plasma Ann Rev Physiol 9 3 7 356 1947
Roberts S and White A Studies on the Origin of Serum Proteins J Biol Chem 180 (ـ) 505 516 (Sept) 1949

Protein

Hiller A Greif R L and Beckman W W Determination of Prote n in Urine by the Biuret Method J Biol Chem 176 14°1 14°9 (Dec) 1948

Purines

Hitchings G H and Fiske C H The Determination of the Purines J Biol. Chem 140 (°) 491 49° (Aug) 1941
Hunter D and Givens H S The Metabolism of Endogenous and Exogenous Purines in the Monkey J Biol Chem 17 37 53 (Feb) 1914
Soodak M Pircio A and Cerecedo L R A Colorimetric Method for the Estimation of Uracil and Cytosine J Biol Chem 181 713 718 (Dec) 1949

Total Nitrogen

Carpenter A M Dilution of Capillary Blood in the Mi roanalysis of Protein Am J Clin Path 17 (7) 565 68 (July) 1947
Kirk P L Kj ldahl Method for Total Nitrogen Anal Chem 22 ( ) 354 358 (Feb) 1950

Tryptophan

Albanese A A Davis V I and Lein M The Utilization of d Amino Acids by Man VIII Tryptophan and Ace yltryptophan J Biol Chem. 172 (1) 39 44 (Jan) 1948

Tryptophane

Alban se A. A and Frankston J E Est mation of Tryptophane in Human Urine J Biol Chem. 157 59 68 (Jan) 1945
Berg C P and Rohe W G The Tryptophane Content of Normal Human Urine J Biol Ch m 170 , , 5 / 9 (Oct) 194
Carpenter D C Spectrophotomet ic Estimation of Tryptophan Anal Chem 20 530 38 (June) 1948
Gordon M and M t hell H. K Fluorometric Method fo the E timat on of Tryptophan J Biol. Chem. 180 (3) 1065 1070 (Oct) 1949
Spies J R and Chambers D C Chemical Det rmination of Tryptophane Anal. Chem 20 30 3 (Jan) 1948
Steer E and S vag M. G Micromethod for Determination of Tryptophane in Bacteria and Protein Anal Chem 21 641 64 (May) 1949

Tyrosine

Goodwin T V and Morton R A The Spe trophotometri D termination of Tyrosine and Tryptophane in Proteins Bio h m J 40 628 63 1946

Urea

Folin O and Svedberg A An Improved Dist llat on Method for the Determination of Urea in Blood J Biol Chem 88 7 83 (Aug) 1930
Obermer E and Milton R Further N tes on a New Color metric Techni que for the Estimation of U ea in Urine Analyst 63 4 3 4 4 (May) 1938
Van Slyke D D and Cullen G E A Permanent Preparation of Urease and Its Use in the Determination of Urea J Biol Chem 19 211 8 (Sept) 1914
Van Slyke D D and Cullen G E The Determinat on of Ur a by the Urease Method J Biol Chem 24 117 1 9 (Jan) 1916

Uric Acid

Blo k W D and Geib N C An Enzymatic Method for the Determination of Uric Acid in Whole Blood J Biol Chem. 168 ( ) 74 756 (May) 1947
Bulger H A and Johns H F The Determinati n of Plasma Uric Acid J Biol Chem 140 427-440 (Aug) 1941
Folin O Standardized Methods for the Determination of Uric Acid in Unlaked B ood and n Urine J Biol Chem 101 111 125 (June) 19
Fol n O The Preparation of Sodium Tungstat Fr e From Molybdate Together With a S mplified Pro ess for the Preparation of a Correct Uric Acid Reagent J Biol Chem 106 311 314 (Aug) 1934
Schaffer N K The De erm nation of Uric Acid in Urine With Crud Uricase J Biol. Chem 153 163 166 (April) 1944
Wolfson W Q Huddlestun B and Levine P The Transport and Excretion o Uric Acid in Man II The Endogenous Uri A id lik Chromog n of Biological Fluids J Cl n. Investigation 26 (5) 995 1001 (Sept) 1947

SECTION IV

BIOCHEMICAL PROCEDURES (Continued)

C CARBOHYDRATES AND DERIVATIVES

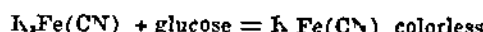
1 Blood Glucose (Colorimetric)

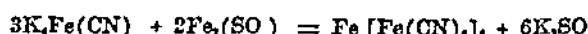
Reference

Folin O and Malmros H An Improved Form of Folin's Micro Method for Blood Glucose Determination J Biol Chem 83 115 120 (July) 1929

Principle

The glucose is oxidized with alkaline ferricyanide and the ferrocyanide produced is measured colorimetrically as Prussian blue Gum ghatti is added to keep the Prussian blue in colloidal suspension So intense is the resulting color that two separate determinations can be made on the extract from 0 1 ml of normal blood. The final color is a mixture of the yellows of the excess ferricyanide and ferric sulfate with the blue of the Prussian blue


$K_3Fe(CN)_6$ + glucose = $K_4Fe(CN)_6$ colorless


$3K_4Fe(CN)_6 + 2Fe_2(SO_4)_3 = Fe_4[Fe(CN)_6]_3 + 6K_2SO_4$

Prussian blue

Apparatus

1 Coleman Junior Spectrophotometer Model 6
2 Cuvettes 19 x 150 mm
3 Tubes blood sugar type graduated at 12.5 and 25 ml
4 Centrifuged tubes 15 ml round bottom
5 Micro pipettes, 0 1 ml These pipettes are calibrated to be washed out
6 A water bath with boiling water
7 An electric centrifuge
8 A wire rack to hold the tubes while boiling

Reagents

1 *Sulfuric acid A 2/3 N solution made by diluting 6[illegible] ml of concentrated H_2SO_4 to 360 ml with water*
2 Sodium tungstate A 10% solution in water
3 Dilute tungstate solution Transfer 20 ml of 10% sodium tungstate solution into a liter volumetric flask Dilute to about 800 ml and while shaking add [illegible] ml 2/3 N H_2SO_4 Dilute to 1000 ml with water
4. Potassium ferricyanide solution $K_3Fe(CN)_6$ Dissolve 2 gm of pure potassium ferricyanide (recrystallized) in 500 ml flask and dilute to mark Keep in dark bottle away from light
5 Sodium carbonate Dissolve 8 gm of sodium carbonate in 500 ml of water
6 Ferric iron solution Fill a liter cylinder to the mark with cold tap water Push into this cylinder a circular piece of copper screening large enough to form a bowl well below the surface of the water Place in it 20 gm gum ghatti #

Cover cylinder and set as de for 18 to 24 hours At the end of this time remove screen and undissolved residue Strain through double thickness of towelling to remove dirt. By aid of heat dissolve 5 gm of anhydrous ferric sulfate in 75 ml of 85% phosphoric acid and 100 ml distilled water Cool and mix with gum ghatti To the final mixture add 1% potassium permanganate solution at first 5 ml at a time and later 3 ml at a time until the pink color remains perceptible for at least three minutes This step is essent al for oxidation of certain ma terials in the gum ghatti wh ch reduces ferricyanide Precaution Avoid excess of $KMnO_4$ which leads to high and erratic results

7 Standard stock glucose solution Dissolve gm of benzoic acid in about 500 ml of hot water in a l ter flask Weigh out exactly 2 0 gm of anhydrous glu *cose* (Bureau of Standards) and place in the flask Dilute to about 900 ml and cool Then dilute to volume The concentration of this solution is 2 gm/l ter or 2 mg/ml

8 Working glucose solution standard D ssolve 2 gm of benzoic acid in about 500 ml of hot water Add 10 ml of stock standard to a liter flask Then add the hot benzoic acid solution and dilute to about 1500 ml When cool dilute to mark This solution contains 0 01 mg of glucose per ml Store in icebox

Procedure

1 Measure accurately 0 1 ml blood in a centrifuge tube containing 10 ml of dilute tungstic acid Rin out pipette 8 times Stopper and shake tube vigorously

2 Centrifuge 3 5 m nutes at 1500 r p m

3 Pipette out exactly 4 ml of filtrate into a tube graduated at 1 5 and 5 ml

4 Add ml of 0 4% potas ium ferricyanide

5 Add 1 ml of sodium carbonate solution just before boiling

6 At the same time set up tubes containing 4 ml of water for a blank and 4 ml of working glucose solut on as a check on the standard curve Treat as in Steps 4 and 5

7 Heat in bo ling water for exactly 8 minutes and cool as soon as possible in run ning cold water 1 or m nutes

8 Add 5 ml of fe ic iron alongside tube to prevent foam ng

9 Mix and after minutes dilute to ml mark

10 Set blank at 100% T at 5 0 mμ wave length and read standa d and unknowns

Calculation

1 Set up a calibration curve with glucose at intervals in the range 0 01 0 10 mg glucose in cuvette sett ng blank at 100% T wave length 5 0 mμ

mg glucose/100 ml blood =

$$\frac{(\text{mg glucose in cuvette}) \times (\text{dilution}) \times 100}{(\text{ml aliquot}) \times (\text{ml blood sample})}$$

Example

A reading of 50 5% T was equivalent to 0 04 mg glucose n the cuvette Samples of blood were 0 1 ml diluted to 10 0 ml of which 4 ml were used for analysis (The precipitate is about 0 1 ml containing very little glucose)

$$\text{mg glucose/100 ml blood} = \frac{(0\ 042) \times (10) \times (100)}{(4) \times (0\ 1)} = 105\ \text{mg/100 ml}$$

Precautions

1 Be sure the water is boiling before adding the sodium carbonate

2 The bo ling time is exactly 8 minutes

3 The *micro pipettes* must be washed out at least 8 t mes to get out all the blood

2 Glucose (Volumetric)

References

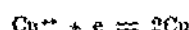
Shaffer P A and Hartman A F The Iodometric Determination of Copper and Its Use in Sugar Analysis J Biol Chem 45 349 390 (Jan) 1921

Somogyi M Notes on Sugar Determinations J Biol Chem 70 599-612 (Nov) 1926

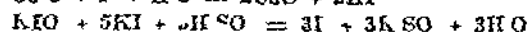
Somogyi M A New Copper Reagent J Biol Chem 160 61 68 (Sept) 1945

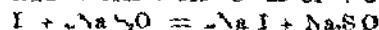
Principle

The chief advantage of this method is its usefulness when samples contain widely varying amounts of glucose Being titrimetric it covers a very wide range without necessitating extra dilutions Cupric ions are reduced to cuprous in a hot medium In acid solution iodine is released stoichiometrically from an iodide iodate mixture and is titrated with thiosulfate


$Cu^{++} + e \rightleftharpoons 2Cu$


$Cu\ O + I + H\ O \rightleftharpoons 2CuO + 2HI$


$KIO + 5KI + 3H\ SO \rightleftharpoons 3I + 3K\ SO + 3H\ O$


$I + 2Na\ S\ O \rightleftharpoons 2Na\ I + Na_2S\ O$

Apparatus

1 Filter paper quantitative
2 A number of special sugar tubes (see references)
3 Boiling water bath
4 *A calibrated burette 10 ml in capacity*
5 A quantity of 15 ml centrifuge tubes (round bottom) with #1 solid rubber stoppers

Reagents

1 Phosphate tartrate copper iodide solution Dissolve 28 gm disodium phosphate in 600 ml water Add 40 gm Rochelle salts dissolve and add *100 ml of 1 N* sodium hydroxide Add 80 ml of a 10% solution of cupric sulfate and with constant stirring 180 gm anhydrous sodium sulfate Dilute to one liter with distilled water and add 8 gm potassium iodide and 25 ml of 3 5% potassium iodate
2 *Sodium thiosulfate* Prepare and standardize 0 1000 N thiosulfate as described in the section on volumetric analysis As needed prepare 0 005 N thiosulfate by diluting 25 ml of 0 1 N to 500 ml
3 *Starch indicator* *To 50 ml of boiling water add 1 gm of* soluble starch and stir until dissolved Then dilute to 100 ml with cold water To prevent molds dissolve 1 gm of salicylic acid in the water before using it in the starch solution
4 Sulfuric acid *Dilute 20 ml of concentrated sulfuric acid to 360 ml* with distilled water This is approximately 2 N
5 Zinc sulfate A 5% solution in water
6 Barium hydroxide An 0 3 N solution is prepared by *dissolving 5 0* gm $Ba(OH)_2$ in 1000 ml warm water Titrate against zinc sulfate These two solutions must be equal in strength

Procedure

1 To a 15 ml centrifuge tube add 4.5 ml of barium hydroxide solution
2 Add exactly 1 ml of blood
3 *Then add 4 5 ml of* zinc hydroxide stopper and shake vigorously
4 Filter
5 Pipette exactly 2 ml of the filtrate into a special sugar tube
6 *Add 2 ml of copper reagent*
The blank consists of 2 ml of distilled water plus 2 ml of copper reagent

8 Cover both tubes and heat in a boiling water bath for exactly 10 minutes Cool under running water
9 Add 1 ml of sulfuric acid rapidly to each tube while mixing gently
10 Add 1 to 2 drops of starch indicator and titrate with 0 005 N sodium thiosulfate The end point is the disappearance of the blue color

Calculation

Addition of iodide to the copper reagent increases the speed of this method but cuts down its accuracy in the titration range 0 to 1 ml Above a titration figure of 1 ml 1 ml of 0 005 N thiosulfate is equivalent to 0 135 mg glucose However to save time in computation it is best to use a table derived empirically for the first ml (Table 9)

Example

The blank titration was 0 12 ml the unknown titration was 1 6 ml One ml of blood was diluted to 10 ml of which 2 ml aliquots were analyzed. Reference to Table 9 shows that (unknown – blank) i e (1 6 – 0 12) is equivalent to 110 mg glucose per 100 ml blood

Precautions

1 Strict attention to every detail of this method is required Especially important are
   a Timing
   b Avoidance of reoxidation of cuprous compounds once they are formed
   c Temperature

TABLE 9

BLOOD GLUCOSE SHAFFER HARTMAN METHOD

(Direct conversion of titration ml 0 005 N thiosulfate into blood glucose when 2 ml aliquots of a 1 10 dilution are analyzed)

ml UNK MINUS ml BLK	0	0 1	0 2	0 3	0 4	0 5	0 6	0 7	0 8	0 9
	mg GLUCOSE/100 ml WHOLE BLOOD									
0	0	11	16	2[illegible]	3	40	47	54	61	6[illegible]
1	5	8	89	96	103	110	117	1[illegible]4	131	137
2	143	149	155	16[illegible]	1[illegible]3	1[illegible]	18	189	1[illegible]5	20
3	09	[illegible]15	[illegible] 1	8	34	41	48	[illegible]4	61	69
4	75	81	288	[illegible]	30	309	315	3[illegible]	3 9	336
5	34	34[illegible]	356	363	370	376	[illegible]83	389	3[illegible]6	403
6	40[illegible]	416	4	4 9	4 6	443	44[illegible]	456	46[illegible]	46[illegible]
7	476	48	489	496	[illegible]03	[illegible]10	516	[illegible] 3	5 [illegible]	536
8	543	549	5[illegible]6	563	570	7	[illegible]83	590	59[illegible]	603
9	610	616	6 3	630	6 7	644	6[illegible]0	657	663	6 0

3 Lactic Acid

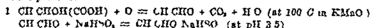
A. IN BLOOD AND SERUM

Reference

Edwards H T A Simplified Estimation of Lactate in Normal Human Blood *J Biol Chem* 125 571 583 (Oct) 1938

Principle

Lactic acid is oxidized to acetaldehyde which is volatile The acetaldehyde is absorbed by bisulfite and the bound bisulfite is estimated iodometrically The steps in the procedure are


1 $CH_3CHOH(COOH) + O = CH_3CHO + CO_2 + H_2O$ (at 100 C in $KMnO_4$)
$CH_3CHO + NaHSO_3 = CH_3CHO \cdot NaHSO_3$ (at pH 3 5)

2 Glucose (Volumetric)

References

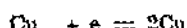
Shaffer P A and Hartman A F The Iodometric Determination of Copper and Its Use in Sugar Analysis J Biol Chem 45 349 390 (Jan) 1921

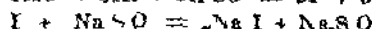
Somogyi M Notes on Sugar Determinations J Biol Chem 70 599 612 (Nov) 1926

Somogyi M A New Copper Reagent J Biol Chem 160 61 68 (Sept) 1945

Principle

The chief advantage of this method is its usefulness when *samples contain widely* varying amounts of glucose Being titrimetric it covers a very wide range without necessitating extra dilutions Cupric ions are reduced to cuprous in a hot medium In acid solution iodine is released stoichiometrically *from an iodide iodate mixture* and is titrated with thiosulfate



Apparatus

1 *Filter paper quantitative*
2 A number of special sugar tubes (see references)
3 Boiling water bath
4 *A calibrated burette 10 ml in capacity*
5 A quantity of 15 ml centrifuge tubes (round bottom) with #1 solid rubber stoppers

Reagents

1 *Phosphate tartrate copper iodide solution* Dissolve 28 gm disodium phosphate in 600 ml water Add 40 gm Rochelle salts dissolve and add 100 ml of 1 N sodium hydroxide Add 80 ml of a 10% solution of cupric sulfate and with constant stirring 180 gm anhydrous sodium sulfate Dilute to one liter with distilled water and add 8 gm potassium iodide and 25 ml of 3 5% potassium iodate
2 *Sodium thiosulfate Prepare and standardize* 0 1000 N thiosulfate as described in the section on volumetric analysis As needed prepare 0 005 N thiosulfate by diluting 25 ml of 0 1 N to 500 ml
3 Starch indicator To 50 ml of boiling water add 1 gm of soluble starch and stir until dissolved Then dilute to 100 ml with cold water To prevent molds dissolve *1 gm of salicylic acid in the water before using it in the starch solution*
4 *Sulfuric acid Dilute* 20 *ml of concentrated sulfuric acid to* 360 ml with distilled water This is approximately 2 N
5 Zinc sulfate A 5% solution in water
6 Barium hydroxide An 0 3 N solution is prepared by dissolving 25 (0) gm $Ba(OH)_2$ in 1000 ml warm water *Titrate against zinc sulfate These two solutions* must be equal in strength

Procedure

1 To a 15 ml centrifuge tube add 4 5 ml of barium hydroxide solution
2 *Add exactly 1 ml of* blood
3 Then add 4 5 ml of zinc hydroxide stopper and shake vigorously
4 Filter
5 Pipette exactly 2 ml of the filtrate into a special sugar tube
6 Add 2 ml of copper reagent
7 The blank *consists of 2 ml of* distilled water plus 2 ml of copper reagent

8 Cover both tubes and heat in a bo l ng water bath for exactly 10 m nutes Cool under runn ng water

9 Add 1 ml of sulfuric ac d rap dly to each tube wh le mix ng gently

10 Add 1 to " drops *of starch* ind cator and t trate w th 0 005 N sod um th osulfate The end po nt s the d sappearance of the blue color

Calculation

Add t on of od de to the copper reagent increases the speed of th s method but cuts down ts accu acy in the t trat on range 0 to 1 ml Above a t trat on figure of 1 ml 1 ml of 0 005 N th osulfate is equ valent to 0 135 mg glucose However to save time n computat on t s best to use a table derived empir cally for the first ml (Table 9)

Example

The blank t trat on was 0 1 ml the unknown t trat on was 1 6 ml One ml of blood was d luted to 10 ml of wh ch ml aliquots were analyzed. Reference to Table 9 shows that (unknown blank) e (1 6 0 1) s equ valent to 110 mg glucose pe 100 ml blood

Precautions

1 Str ct attent on to every deta l of th s method s requ red Espec ally mportant are

a T m ng

b Avo dance of eox dat on of cuprous compounds once they are formed

c Temperature

TABLE 9

BLOOD GLUCOSE SHAFFER HARTMAN MET OD

(D rect convers on of titrat on ml 0 005 N th osulfate nto blood glucose when ml al quots of a 1 10 dilut on are analyzed.)

ml UNK MINUS ml BLK	0	0 1	0 2	0 3	0 4	0 5	0 6	0 7	0 8	0 9
	mg GLUCOSE/100 ml WHOLE BLOOD									
0	0	11	18	25	3	40	47	54	61	68
1	75	8	89	96	103	110	11	1 4	131	137
2	143	149	1 5	16	169	175	18	189	195	0
3	09	215	1	2 8	234	41	48	54	61	68
4	2 5	81	288	95	30	309	315	3	3 9	336
5	342	349	356	363	370	376	383	389	396	403
6	409	416	4	4 9	436	443	449	456	46	469
7	476	482	489	496	503	510	516	5 3	5 9	536
8	543	549	556	563	5 0	5 7	583	590	596	603
9	610	616	6 3	630	637	644	650	657	663	6 0

3 Lactic Acid

A. IN BLOOD AND SERUM

Reference

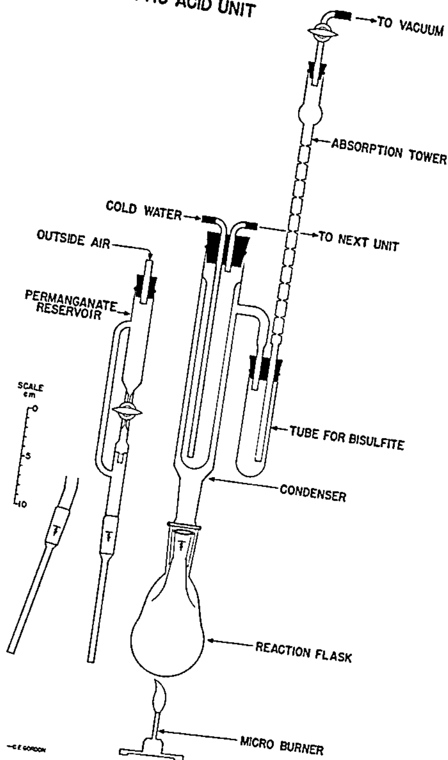
Edwards H. T A S mpl fied Est mat on of Lactate n Normal Human Blood *J B ol Chem* 125 571 583 (Oct) 1938

Principle

Lact c ac d is ox d zed to acetaldehyde wh ch is volat le The acetaldehyde is ab sorbed by b sulfite and the bound b sulfite s est mated iodometr cally The steps n the procedure are

1 CH CHOH (COOH) + O = CH CHO + CO + H O (at 100 C in KMnO)

2 CH CHO + NaHSO — CH CHO NaHSO (at pH 3 5)



gram of the apparatus to scale. The reaction flask is 15 cm its capacity 5 ml.

3 $HSO_3^- + 2I + H_2O = HSO_4^- + 2HI$
4 $CH_3CHO \cdot NaHSO_3 = CH_3CHO + NaHSO_3$ (at pH 8 9)
5 $HSO_3^- + I_2 + H_2O = HSO_4^- + 2HI$

Apparatus

1 Special lactic acid apparatus (See Fig 21)
2 50 ml centrifuge tubes
3 Electric centrifuge
4 0 5 ml pipettes or accurately calibrated syringe pipette
5 Micro burette 3 ml accurately calibrated

Reagents

1 Sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) approximately 10% Adjust to pH 8 9 using thymol blue as an indicator with dilute NaOH or H_2SO_4. (High blanks can sometimes be avoided by boiling 1 liter of this with 0 25 ml of 3% H_2O_2)
2 Sulfuric acid (H_2SO_4) 2/3 N made from special concentrated Pregl acid 6 6 ml H_2SO_4 made up to 360 ml of water
3 Manganous sulfate ($MnSO_4 \cdot H_2O$) approximately 10%
4 Sulfuric acid approximately 10 N made from special Pregl sulfuric acid (to get a low blank) in distilled water 100 ml is made up to 360 ml with water (Concentrated acid is 36 N)
5 Sodium meta bisulfite ($Na_2S_2O_5$) approximately 5% Keep in glass stoppered bottle Do not use after two weeks
6 Potassium permanganate ($KMnO_4$) approximately N/200
7 Starch solution approximately 1%
8 Sodium meta bisulfite ($Na_2S_2O_5$) 0 05% Make fresh daily
9 Iodine (I_2) approximately N/3 3 gm I and 48 gm of KI made up to 1 liter Use crude iodine and USP KI Add more KI if necessary to get the iodine in solution
10 Iodine approximately N/10 Dilute from above
11 Iodine standard N/200 made by diluting standard N/10 iodine N/200 is stable for 2 3 days. N/10 is stable for several months Make up and standardize N/10 iodine
12 Disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) ground to a powder Use Eimer and Amend sodium phosphate secondary USP granular
13 Standard lithium lactate $CH_3CHOHCOOLi$ mol wt 95 940 Prepare the salt according to Friedemann and Graeser (J Biol. Chem 100 291 308 March 1933) Prepare an accurate 0 1 M solution in 0 2 N H_2SO_4. Dilute this stock standard 1 to 500 with 0 1 N H_2SO_4 The diluted standard keeps well in the refrigerator Of this 15 ml is equivalent to 0 270 mg lactic acid or 1 0 ml N/200 iodine

Procedure

This method is also valid for trichloroacetic acid centrifugates from blood and muscle Once the blood is hemolyzed it can be kept in the icebox at any stage of precipitation the value being constant for a week or more It is best to remove the filtrate from precipitate if it will not be analyzed within a few days

1 For whole blood pipette exactly 1 ml of whole blood into 45 ml of distilled water For plasma pipette 1 ml plasma into 17 ml of distilled water Mix For cells pipette 1 ml of cells into 53 ml of distilled water Mix For cells it is best to use a pipette calibrated to contain and wash out
2 Add mixing between additions
For whole blood pipette 2 ml of 2/3 N sulfuric acid and 2 ml of 10% tungstate
For plasma 1 ml of 2/3 N sulfuric acid and 1 ml of 10% tungstate
For cells 3 ml of 2/3 N sulfuric acid and 3 ml of 10% tungstate
3 Centrifuge 5 10 minutes at 3000 r p m *Note*
a If alcohol is present boil the filtrate for 5 minutes with new reagents in Steps 4 8 before adding permanganate

b If ether is present bubble cold air through the filtrate *15 minutes before using*

c In mild ketosis use the filtrate just as with normal blood

4 Clean absorption towers and test tubes with distilled water

5 *Pipette 10 or 15 ml aliquots of the centrifugate into the reaction flasks* (Lift up the $KMnO_4$ reservoir 6 inches, insert pipette lower reservoir to hold it, and allow pipette to drain while you do the other pipettings This saves time) For standard lactate use about 0 2 mg of lactate

6 Add approximately 5 ml 10 N sulfuric acid approximately 10 ml of 10% $MnSO_4$ and approximately 50 ml distilled water to each reaction flask Mix these three in graduate and add all together When first run is finished add fresh unknown solutions directly to the used solutions in the reaction flasks even when the old $MnSO_4$ H_2SO_4 solutions are deep brown or hot, or both As many as 9 subsequent runs can be made without addition of fresh reagents or cleaning out the flasks (Add 0 5 ml 10 N H_2SO_4 every 2 4 runs)

7 Add approximately 1 5 ml 5 % $NaHSO_3$ to each absorption tube but not through tower adjust absorption tower *fill air intake reservoir with $KMnO_4$ (N/ 00)* (approximately 9 ml) with stopcocks shut and connect all the rubber stoppers

8 Make sure water in condenser is flowing Adjust the air to flow briskly (The exact rate does not matter provided it is brisk) Light the burners bring to *boiling point and add $KMnO_4$ by adjusting stopcocks to drip so that after 10* minutes a faint pink shows in each flask. About 1 drop every 5 seconds is needed If $KMnO_4$ collects at dripping point a vigorous shake after the run will dispel the collection (In subsequent runs a brown precipitate obscures the pink color but drop in the $KMnO_4$ at the usual rate) Be sure to add all of the $KMnO_4$ in dropping reservoir in order to oxidize all unknown

9 Add $KMnO_4$ for a total of exactly 15 minutes Continue boiling for 5 additional minutes

10 At the end of the 20 minutes turn off the burners and turn off the air flow Loosen one tube at a time and wash the tower while holding the tube with the tip of the tower near the top of the tube

11 Wash the towers with four 2 ml portions of distilled water from a fast running 2 ml pipette Do this by washing with the first portion around the top of the absorption tower and with the next three so as to cover ½ of the tower each time Wash the tip of the tower with about 1 ml of water

Titration

Note If room temperature is above 24 , the tubes must be kept cold in a pan of ice

1 Add 4 drops of starch to the tube

2 Add strong iodine first then 0 05 N iodine until a blue color persists (Avoid *adding a large excess of I because this raises the blank There is no need to hurry with the titration at this point*)

3 Add 0 05% $NaHSO_3$ until the blue color is discharged (Clean this burette with distilled water and add fresh 0 05% $NaHSO_3$ each day)

4 Add 0 005 N iodine until a faint blue color persists 15 seconds When you think you have reached the end point tip the tube so that the contents rinse down the sides This prevents drops of iodine or $NaHSO_3$ on the sides of the tube giving *bad results Be sure the end point is still present after tipping*

5 Add 2 spatulas full of Na_2HPO_4 $12H_2O$ to discharge the blue color and mix thoroughly by swirling the tube

6 Read the burette containing the standard N/ 00 iodine

7 Add N/200 iodine until the first *faint blue color* that persists for 15 seconds agitate the tube constantly It does not matter if this end point fades after 15 seconds

8 Read the burette again.

9 Subtract the readings

10 Subtract the blank from this result (The blank should never be over 0 04 ml of 0 005 N iodine) It is well if possible to run a distilled water blank and a standard lactate solution with each run The reagent blank should be the same as a water blank If it is not change the reagents The commonest sources of high blanks are the H SO the Na WO and the excess iodine during titration The blank appears to be due to the dist lled water used

11 Duplicate titrations must agree to 0 0 ml of 0 005 N I_2

Calculation

mg lactic ac d/100 ml blood =

$$\frac{\begin{pmatrix}\text{ml titration}\\ -\text{ ml blank}\end{pmatrix} \times \begin{pmatrix}\text{normality of}\\ \text{iodine}\end{pmatrix} \times (45) \times (\text{dilution}) \times 100}{(\text{ml aliquot}) \times (\text{ml blood sample})}$$

Example

The standard iodine was 0 0049 N One ml of whole blood was d luted to 50 ml of which 15 ml al quots were used The average titration was 1 09 ml and the blank 0 04 ml of iodine

mg lactic acid/100 ml blood =

$$\frac{(1\ 09 - 0\ 04) \times 0\ 0049 \times 45 \times 50 \times 100}{15 \times 1} = 77\ 2 \text{ mg/100 ml}$$

Precautions

1 There are many steps in this method three of which (oxidation binding of aldehyde titration) are subject to many errors Most te hnicians need a great deal of practice before the method runs smoothly

2 Clean ng the apparatus is best done by stand ng in a mixture of 4 vols con cent ated HCl + 1 vol of 5% NaHSO until the precipitated MnO is dissolved Then wash thoroughly with cold tap water and rinse off with distilled water Precipitates of tungstic oxide can be removed with d lute NaOH The burette containing 0 05% NaHSO and containing 0 005 N I must be rinsed out with distilled water each day The burette containing more concentrated iodine may be left until its contents have been completely used

B LACTIC ACID IN MUSCLE

Preparation of Specimen

1 Set the tube of a homogen zer tube in a pan of sol d CO

Pipette in 2 ml of 10% trichloracet c acid and allow to freeze

3 The muscle or tissue (approximately 250 mg) is we ghed on a Roller Smith type balance and frozen in powdered CO

4 It is then dropped into the test tube covered with 3 ml more of trichloracetic acid and allowed to freeze solidly

5 The tube is dipped in cold water until melting just begins the pestle is inserted the motor started and the muscle s ground to a fine pulp This takes about 2 minutes

6 The pestle is withdrawn and washed off quantitat vely into the test tube with ice cold trichloracetic ac d

7 Dilute to the 25 ml mark insert a clean rubber stopper and shake

8 Centrifuge for 5 minutes at 00 r p m

9 Pipette out an al quot and proceed as for whole blood

4 Glycogen in Tissue

Reference

Good C A Kramer H and Somogyi M The Determination of Glycogen J B ol Chem 100 485 491 (April) 1933

Association of Official Agricultural Chemists Methods of Analysis ed 5 Washing ton D C 1940 Assoc of Offic al Agr cultural Chemists

Principle

The tissue is hydrolyzed with boiling KOH which does not destroy glycogen The glycogen is precipitated with ethyl alcohol and is hydrolyzed with boiling HCl Sugar is then estimated by any appropriate macro method such as that of Shaffer Hartman Somogyi.

Apparatus

1 Conical 15 ml graduated centrifuge tubes
2 Hollow glass balls which fit the necks of the centrifuge tubes These act as reflux condensers

Reagents

1 Potassium hydroxide (KOH) approx 5 4 N
2 Potassium hydroxide approx 0 01 N
3 Hydrochloric acid (HCl) 0 55 N For this solution take 31 4 ml of 35% acid and dilute it to 500 ml with distilled water
4 Thymol blue approx 1% aqueous solution
5 Ethyl alcohol (EtOH) 95%
6 Glycogen standard solution 80 mg/100 ml Weigh out 0 08 gm glycogen and dissolve in 100 ml of 20% KOH solution This solution is stable in icebox for at least one week
7 Shaffer Hartman Somogyi sugar reagents

Procedure

1 Place 1 2 ml (enough to cover the muscle) of either cold or hot 20% KOH in the tube fitted with the air condenser
2 After weighing the frozen muscle (about 0 5 gm) drop it into the tube immediately replace the air condenser and place the tube in a boiling water bath Boil for 30 minutes occasionally removing the tube and shaking it to aid the hydrolysis
3 Cool the tube to room temperature and add 95% alcohol The volume added should equal 1 1 to 1 2 times the volume of solution already in the tube *Stir the solution vigorously with a platinum wire until a complete mixing results* Any glycogen will begin to precipitate here Place the tube in warm water Slowly and carefully bring the alcoholic solution to the boiling point and immediately remove the tubes from the water bath Cool to room temperature This completes the coagulation of the glycogen
4 After the precipitate has been allowed to settle somewhat note carefully the amount present This value will be used later in the procedure
5 Remove the condensers and centrifuge the tube at the maximum speed for 15 minutes Decant the solution and drain the tubes for a few minutes *If the rest of the estimation must be delayed this is the best place to stop Place the* tubes inverted in the icebox until the estimation can be resumed.
6 Remove the remaining alcohol by placing the tubes in boiling water or in an oven at 100 for about 5 minutes or until no more alcohol can be detected by its odor This is not necessary if the tubes have stood in *the icebox overnight*
7 Cool and add 2 2% HCl in amounts according to the amount of precipitated glycogen noted earlier

Use about 6 ml HCl for	0 3 0 4 ml ppt
Use about 5 to 5½ ml HCl for	0 2 0 3 ml ppt
Use about 4 ml HCl for	0 1 0 2 ml ppt

Never use less than *1.5 to 2 ml* HCl Place the tubes with attached air condensers in boiling water and hydrolyze for 2½ hours. Cool to room temperature

8 Add 1 drop of thymol blue to each tube If the solution is not to be estimated for over 5 hours adjust the pH in the range of 2 to 6 by adding KOH from a dropper until the indicator is yellow in color and place the covered tubes in the icebox until the determination can be resumed Then adjust the pH of the solution to 8 with 30% KOH 0 5% KOH and 2 N HCl the end point is reached when the indicator has just reached a pale purple color

9 Estimation of glucose by Shaffer Hartman Somogyi method This method is recommended because it covers a wide range of glucose concentrations without necessitating many dilutions

a Adjust the pH to 8 as in Step 8

b Dilute to a measured volume e g 5 ml and use aliquots containing 0 05 1 50 mg glucose The volume of aliquot should be around 5 ml

c Measure glucose by the Shaffer Hartman Somogyi method

10 Estimation of Glucose by Method of Folin and Wu This method may be preferred by those who use it routinely for blood

a Adjust pH to 8 as in Step 8

b Dilute the solution to a known volume with distilled water according to the amount of precipitated glycogen noted earlier If the precipitate was greater than 0 4 ml dilute to 15 ml and use 1 ml of the solution and 1 ml of distilled water for the sugar determination

If ppt was 0 3 0 4 ml make up to 15 ml and use 2 ml
If ppt was 0 2 0 3 ml make up to 1 15 ml and use 2 ml
If ppt was 0 1 0 2 ml make up to 8 10 ml and use 2 ml
If ppt was 0 1 or less make up to 6 8 ml and use 2 ml

c Proceed as described under the method of Folin and Wu

Calculation

$$\text{mg glucose/100 gm tissue} = \frac{(\text{mg glucose /ml hydrolysate}) \times (\text{dilution}) \times (100)}{(\text{ml aliquot}) \times (\text{gm tissue})}$$

For the Shaffer Hartman Somogyi method an empirical calibration curve is required Set up glycogen standards at intervals in the range 0 05 mg 1 50 mg/5 ml run through the hydrolysis technique previously described neutralize and estimate the glucose

Example

A sample of muscle weighed 26_ mg After hydrolysis the solution was diluted to 25 ml and 5 ml aliquots were analyzed for glucose The average concentration was found to be 0 16 mg glucose/ml of dilute hydrolysate

$$\text{mg glycogen (as glucose)/100 gm tissue} = \frac{(0\ 16) \times 5 \times 100}{5 \times 0\ 6} = 30_ \text{ mg/100 gm}$$

Precautions

1 The chief source of error in this method is glycogenolysis in the tissue before the glycolytic system can be inhibited or inactivated Extreme care is required in handling the tissue

5 Alcohol in Blood and Urine

Reference

Gibson J G II and Blodner H The Determination of Ethyl Alcohol in Blood and Urine With the Photoelectric Colorimeter J Biol Chem. 126 551 559 (Nov) 1938

Principle

Alcohol is separated from blood filtrate by distillation Alcohol is oxidized by potassium dichromate causing a loss of color



$$3C_2H_5OH + 2K_2Cr_2O_7 + 8H_2SO_4 = 3CH_3COOH + 2K_2SO_4 + 2Cr_2(SO_4)_3 + 11H_2O$$

Apparatus

1 An alcohol *still* (*or a Kjeldahl* ammonia still)
2 A quantity of ₀0 ml round bottom test tubes with a number of #1 solid rubber stoppers
3 An electric centrifuge
4 A quantity of 25 ml glass stoppered graduated cylinders
5 A Coleman Junior Spectrophotometer
6 Cuvettes 19 × 1₀0 mm
7 An accurately calibrated 2 ml syringe pipette

Reagents

1 Potassium di hromate (K Cr O) Dissolve 3 1943 gm pure salt in 1000 ml water
2 Sodium tungstate (Na WO) Dissolve 100 gm to make 1 liter
3 Sulfuric acid (H SO) Prepare 2/3 N H SO by diluting 6 6 ml of concentrated acid to 360 ml with water
4 Sulfuric acid (H SO) Concentrated and 18 N both used
5 Potassium oxalate (K_2C_2O) Prepare a saturated solution in water One drop will prevent clotting in 10 ml of blood
6 *Standard anhydrous aldehyde free ethyl alcohol* Mix together one liter absolute EtOH 0 5 gm NaOH and ’5 gm $AgNO_3$ dissolved in 3 ml water Reflux 2 to 3 hours Distill and store first 800 ml in brown bottle for use as standard

Procedure

1 Pipette exactly 2 ml of oxalated blood into 14 ml water in a 50 ml centrifuge tube
2 Add 2 ml of 2/3 N sulfuric acid mix
3 Add 2 ml of 10% sodium tungstate
4 Mix and centrifuge for 10 minutes
5 Place in a 100 ml Kjeldahl flask 10 ml of blood filtrate For urine use 1 to 10 ml depending on the concentration of alcohol suspected and add water to make 10 ml in the flask
6 Distill off 6 to , ml into 2₀ ml gla s stoppered cylinder containing 5 ml of 18 N sulfuric acid and 2 ml of potassium dichromate The volume in the cylinder should be about 15 ml after distillation The cylinder is cooled in an ice bath during distillation
7 Mix and after sealing glass stopper with a drop of conc *sulfuric acid heat* cylinder and contents in a water bath for 20 minutes at 75 80 C
8 Then cool to room temperature and dilute to exactly °0 ml
9 Mix and decant into a colorimeter tube
10 At the same time prepare a reagent blank by mixing 5 ml 18 N H SO 13 ml water and ° ml of potassium dichromate
11 Set colorimeter at 440 mμ and a water tube at 100% T

Calculation

1 Prepare a calibration curve from standard alcohol solutions containing *0 to 150* mg per 100 ml at intervals of 15 mg per 100 ml and running them through the whole method Note that in this case the higher the % T the more concentrated is the alcohol

2 mg alcohol/100 ml flu d $= \dfrac{(\text{mg alcohol in cuvette}) \times (\text{dilution}) \times (100)}{(\text{ml aliquot}) \times (\text{ml fluid sample})}$

Example

A reading of 60% T was equivalent to 0 15 mg alcohol in the cuvette *During the* analysis 2 ml blood were diluted to 20 ml *of which 10 ml aliquots were analyzed*

$$\text{mg alcohol/100 ml blood} = \frac{(0\ 15) \times {}^{\circ}0 \times 100}{10 \times 2} = 15\ 0 \text{ mg/100 ml}$$

Precautions

1. In this method the commonest sources of error are
   a. Loss of alcohol during distillation
   b. Erratic color from failure to observe conditions of oxidation exactly
2. The alcohol standard is critical, it must be water and aldehyde free

6 Total Bisulfite Binding Capacity

Reference

Thompson, R. H. S., and Johnson, R. E.: Blood Pyruvate in Vitamin B Deficiency. Biochem. J. 29, 674-700 (May) 1935.

Principle

On occasion, as in exercise or in thiamine deficiency, an increase in the keto acids of blood is observed. The present method gives a measure of the increase of total keto acids in the blood by virtue of their capacity to combine with bisulfite in acid solution. These complexes are broken down in alkaline solution and the bisulfite is titrated iodometrically. The present method is not useful in the presence of ketosis.

For specific compounds such as pyruvic acid and alpha ketoglutaric acid the reader should use methods recommended in the bibliography of the succeeding pages.

Apparatus

1. An electric centrifuge
2. An accurately calibrated 3 ml burette
3. A number of [illegible] ml round bottom tubes
4. An ice bath
5. A number of 2[illegible] ml volumetric flasks
6. A number of 15 ml round bottom centrifuge tubes

Reagents

1. Trichloroacetic acid, a 4% solution in water
2. Thymol blue indicator
3. Sodium meta bisulphite, a 5% solution in water
4. Starch indicator (see lactate method)
5. Standard iodine, 0.005 N (see lactate method)

Procedure

1. Pipette 9 ml of 4% trichloroacetic acid into a 15 ml centrifuge tube
2. Add exactly 1 ml of blood and mix by inversion
3. Centrifuge for 5 minutes
4. Transfer 8 ml to 25 ml volumetric flask and adjust pH to [illegible] 3.0 using thymol blue (orange color)
5. Dilute to [illegible]5 ml with water
6. Pipette out 10 ml samples into lactate titration tubes
7. Add 1 ml of sodium bisulfite (5%)
8. Allow to stand at room temperature for 15 minutes
9. Titrate in ice bath as in lactate. The end point is difficult
10. Set up a blank from trichloracetic acid treated as blood filtrate

Calculation

Bisulfite binding capacity expressed as mg pyruvic acid/100 ml blood =

$$\frac{(\text{ml titration} - \text{ml blank}) \times (45) \times (\text{normality of iodine}) \times \text{dilution} \times 100}{(\text{ml aliquot}) \times (\text{ml blood used})}$$

Example

A titration of 0 25 ml and a blank of 0 04 ml were obtained Of blood 1 ml had been precipitated in 10 ml 8 ml of this were diluted to 25 ml and 10 ml aliquots were titrated Iodine was 0 005 N

Bisulfite binding capacity expressed as mg pyruvic acid =

$$\frac{(0\ 25 - 0\ 04) \times 45 \times 0\ 005 \times 25 \times 10 \times 100}{10 \times 8 \times 1} = 14\ 7$$

Precautions

The end point is difficult and titration must be carried out in the cold

7 Carbohydrates Miscellaneous References

Aldehydes

Siggia S and Maxcy W Improved Procedure for Determination of Aldehydes Anal Chem **19** 1023 1025 (Dec) 1947

Citric Acid

Goldberg A S and Bernheim A R Citric Acid Determination J Biol Chem **156** 33 46 (Nov) 1944
Natelson S Lugovoy J K and Pincus J B Determination of Micro Quantities of Citric Acid in Biological Fluids J Biol Chem **170** 597 606 (Oct) 1947
Saffran M and Denstedt O F A Rapid Method for the Determination of Citric Acid J Biol Chem **175** 849 855 (Sept) 1948
Taussky H H A Micro Colorimetric Method for the Determination of Citric Acid II A Note on the Substitution of Ferrous Sulphate for Hydrazine Sulphate as the Reducing Agent J Biol Chem **181** 195 198 (Nov) 1949
Wolcott G H Boyer P D A Colorimetric Method for the Determination of Citric Acid in Blood and Plasma J Biol Chem **172** 729 736

Glucose

Benedict, S R The Analysis of Whole Blood II The Determination of Sugar and Saccharoids (Non fermentable Copper Reducing Substances) J Biol Chem **92** 141 159 (June) 1931
Folin O and Wu H A System of Blood Analysis J Biol Chem **38** 106 110 (May) 1919
Hall D A A Simplified Method of Blood Sugar Estimation Brit. M J pages 351 353 (Feb) 1950
Kingsley G R and Reinhold J G The Determination of True Glucose in Blood by Reduction of Ferricyanide J Lab & Clin Med **34** (5) 713 719 (May) 1949
Polis B D, and Sortwell M *Rapid Photocolorimetric Micro Procedure for Blood Sugar* Using Copper Reduction With Perchloric Acid Deproteinized Filtrates Arch Biochem **11** (2) 9 233 (Oct) 1946
Schales O and Schales S S A Simple Method for Determination of Glucose in Blood Arch Biochem **8** (2) 285 292 (Nov) 1945

Glycogen

Wagner R The Estimation of Glycogen in Whole Blood and White Blood Cells Arch. Biochem **11** (2) 249 58 (Oct) 1946

Inulin

Little J M A Modified Diphenylamine Procedure for the Determination of Inulin J Biol Chem **180** 747 754 (Sept) 1949
Robson J S Ferguson M H Olbrich O and Stewart C P The Determination of the Renal Clearance of Inulin in Man Quart J Exper Physiol **35** *111 134 (June)* 1949
Roe J H Epstein, J H and Goldstein N P A Photometric Method for the Determination of Inulin in Plasma and Urine J Biol Chem **178** 839 845 (April) 1949

Lactic Acid

Friedemann T E and Graeser J B The Determination of Lactic Acid J Biol Chem **100** 291 308 (March) 1933
Milton R Notes on Mendel and Goldscheider s Method for Determining Lactic Acid in Blood Analyst **61** 91 96 (Feb) 1936

Mannitol

Corcoran A C and Page I H A Method for the Determination of Mannitol in Plasma and Urine J Biol Chem 170 165 171 (Sept) 1947

Methylpentose

Dische Z and Shettles L B A Specific Color Reaction of Methylpentoses and a Spectrophotometric Micromethod for Their Determination J Biol Chem 175 595 603 (Sept) 1948

Pentose

Dische Z Spectrophotometric Method for the Determination of Free Pentose and Pentose in Nucleotides J Biol Chem 181 3/9 39 (Nov) 1949

Dunstan S and Gillam A E A Micro Method for the Determination of Pentoses by Photoelectric Spectrophotometry J Chem Soc Suppl Issue No 1 S140 144 1949

Roe J H and Rice E W A Photometric Method for the Determination of Free Pentoses in Animal Tissues J Biol Chem 173 50 51 (April) 1948

Pyruvic Acid

Klein, D The Determination of Pyruvic Acid in Blood in Presence of Acetoacetic Acid J Biol Chem 137 311 316 (Jan) 1941

Tsao M and Brown S Pyruvic Acid Determination A Micro Method J Lab & Clin Med 35 (2) 3 0 3?3 (Feb) 1950

SECTION IV

BIOCHEMICAL PROCEDURES (*Continued*)

D FATS AND DERIVATIVES

1 Total Lipids in Serum

Reference

Bloor W R The Determination of Small Amounts of Lipid in Blood Plasma J Biol Chem 77 53 73 (April) 19?8

Principle

The lipids are extracted with ether alcohol and saponified with sodium ethylate Fatty acids and cholesterol are extracted with petroleum ether and oxidized with silver and potassium dichromate *The excess dichromate is titrated with sodium thio sulfate*

Apparatus

1 A quantity of 25 and 50 ml volumetric flasks
2 A steam bath
3 Special quantitative filter paper (Whatman 40)
4 *A quantity of 125 ml glass stoppered Erlenmeyer flasks*
5 An electric oven set at 124 C

Reagents

1 Ether alcohol mixture 3 volumes of 95% ethyl alcohol (C_2H OH) and 1 volume of ethyl ether (C_2H) O
2 Sodium thiosulfate (Na_2S O) 0 1000 N (See section on volumetric methods.)
3 Potassium iodide (KI) a 10% solution made freshly
4 Starch suspension (See lactate method)
5 Silver dichromate (Ag Cr O) Dissolve 5 gm of silver nitrate in 25 ml of water *Add 5 gm of K Cr_2O dissolved in 50 ml of water Stir and centrifuge* Wash precipitate twice with water to remove NO Dissolve in 500 ml of concentrated H SO
6 Petroleum ether b p 40 60 C
7 Sodium ethylate (C_2H ONa) Introduce ? 3 gm of fresh sodium ethylate into 100 ml of absolute EtOH containing 1 ml of 95% alcohol (EtOH) Cool Store in cool dark place
8 Dilute sulfuric acid To one volume concentrated H SO add 3 volumes of water
9 Potassium dichromate (K_2Cr O) 1 0000 N (See section under standardization)
10 Methyl red indicator (0 1% in water)

Procedure

1 Into 40 ml of the ether alcohol mixture in a 50 ml volumetric flask pipette exactly 3 ml of serum or plasma
2 Let stand 1 hour Make up to 50 ml with ether alcohol
3 Mix Filter through filter paper
4 Add 0 ml of filtrate to 1 5 ml Erlenmeyer flask
5 Add 2 ml of sodium ethylate
6 Evaporate on steam bath to disappearance of alcohol odor Do not overheat
7 Remove last traces of alcohol by stream of air
8 To the residue add 1 ml of dilute sulfuric acid
9 Heat mixture on steam bath for one minute

10 Pour 10 ml petroleum ether onto hot mi ture and allow to come to a boil
11 Rotate flask gently ? 3 minutes
1 Add 1 drop of methyl red to see the separation between ether water layer
13 Pour only hot ether ext act into 25 ml volumetric flask
14 Repeat extractions with 3 to 5 ml port ons of petroleum ether combining the extracts
15 Cool and dilute to 5 ml with petroleum ether Mix
16 Pipette 10 ml aliquots of the extract into 1 5 ml glass stoppered Erlenmeyer flasks
17 Evaporate remove last traces of ether with a stream of air
18 Add 5 ml of Ag Cr O reagent and 3 ml of 1 0000 N K Cr O rotating flask wh le adding reagents
19 Heat loosely stoppered flask at 1 4 C in an electric oven for 0 m nutes a blank b ng treated with Ag Cr O and K Cr O in the same manner
.0 St r solut ons and aga n leave at 124 C for 40 minutes more If at end of 5 minutes the solution is green add more of the dichromates
1 To hot flasks ad 1 75 ml of water
Add 10 ml of KI Do not stir
3 Titrate with th osulfate until the yellow color fades Add starch indicator and titrate to the d sappearance of blue color The flask may be agitated vigorously as the iodine s tak n p by the thiosulfate

Calculation

1 Th s method measures principally palm tic oleic and stearic ac ds and cholesterol A factor of 1 mg total fat = 0 3(0 ml 1 N dichromate has been determined empirically for the mixture typical of plasma
? mg total lip ds/100 ml plasma =

$$\frac{(\text{ml thiosulfate blank} - \text{ml unknown}) \times (\text{dilution} \quad) \times (\text{dilut on } 1) \times (100)}{(3\ 60) \times (\text{ml al quot} \quad) \times (\text{ml al quot } 1) \times (\text{ml plasma used})}$$

Example

Three ml plasma were extracted in 50 ml 20 ml aliquots were sapon fied and diluted to 5 ml and 10 ml aliquots were oxidized T tration of blank was 33 ml 0 1 N th o sulfate that of unknown was 26 0 ml

$$\text{mg total lipids/100 ml pla ma} = \frac{(33 - 6) \times 5 \times 50 \times 100}{3\ 60 \times 10 \times .0 \times 3} - 406 \text{ mg/100 ml}$$

Precautions

Being rather empir cal this method requ res r gid adherence to time temperature spec fications

2 Total Cholesterol

Reference

Modification of Bloor W R The Fatty Acids of Blood Plasma J B ol Chem 56 711 , 4 (July) 19 3

Principle

For some cl nical purposes the quick method recommended here for determining total cholesterol will prove satisfactory Chole terol is extracted into a mixtu e of ether and alcohol wh ch is then evapo ated A greenish blue color is developed from the residue in the pres nce of chloroform acetic anhydride and sulfuric acid

Apparatus

1 A quantity of 15 ml round bottomed test tubes
Cal brated 15 ml conical test tubes

3 0 2 ml accurately calibrated syringe pipette
4 A number of 10 ml pipettes
5 Graduated pipettes
6 Cork and rubber stoppers
7 Coleman Jr Spectrophotometer Model 6
8 Cuvettes 19 × 150 mm
9 An *electric centrifuge*
10 A variety of #4 cork stoppers
11 A boiling water bath

Reagents

1 Mixture of ether and alcohol. 3 volumes of anhydrous ethyl alcohol (C_2H_5OH) and 1 volume of ethyl ether $(C_2H_5)_2O$
2 Chloroform ($CHCl_3$) dry Merck s reagent
3 Acetic anhydride $(CH_3CO)_2O$ Merck s reagent
4 Sulfuric acid (H_2SO_4) conc ACS
5 Stock standard cholesterol 400 mg cholesterol in 100 ml chloroform
6 Working standard cholesterol dilute stock standard 1 to 100 with chloroform 10 ml = 0 4 mg cholesterol

Procedure

1 To 9 8 ml of ether alcohol in a calibrated cone tipped centrifuge tube add exactly 0 2 ml serum Stopper with a rubber stopper
2 Allow to rest on side 60 minutes with occasional shaking or place in a rotator for 30 minutes
3 Adjust volume to exactly 10 ml with ether alcohol mix, install tight cork stopper and centrifuge 5 to 10 minutes Check the volume after centrifuging
4 Place an 8 ml aliquot of the ether alcohol supernatant fluid in a 15 ml round bottomed test tube
5 Evaporate to dryness in a water bath AVOID OPEN FLAME This takes about 10 minutes Cool
6 Add 10 ml chloroform 4 ml acetic anhydride and 0 2 ml sulfuric acid Stopper with rubber stopper Mix
7 Stand 20 minutes at 25 C ± 1 C preferably in water bath
8 At the same time set up a reagent blank starting with steps 1 and omitting serum.
9 Transfer unknowns and blanks to cuvettes and read at 660 mμ with the blank set at 100% T

Calculation

1 Set up a calibration curve covering the range 0 to 0 5 mg cholesterol in the cuvette with samples of standard cholesterol run through the whole method

2 $$\text{mg cholesterol/100 ml serum} = \frac{(\text{mg cholesterol in cuvette}) \times (\text{dilution}) \times 100}{(\text{ml aliquot}) \times (\text{ml serum used})}$$

Example

A reading of 72% T was equivalent to 0 15 mg cholesterol in the cuvette For analysis 0 2 ml of serum was extracted in 10 ml solvent, of which 8 ml was analyzed

$$\text{mg cholesterol/100 ml serum} = \frac{(0\ 15) \times 10 \times 100}{8 \times 0\ 2} = 93\ 7\ \text{mg/100 ml}$$

Precautions

1 This method is recommended only for clinical purposes not for cases in which high precision and fractionation are required
2 Beware of open flames at all stages

3 Free and Combined Cholesterol in Blood

References

Schoenheimer R and Sperry W M A Micro Method for the Determination of Free and Combined Cholesterol J Biol Chem 106 ,45 760 (Sept) 1934

Sperry W M and Brand F C The Colorimetric Determination of Cholesterol J Biol Chem 150 315 4 (Oct) 1943

Principle

Cholesterol exists in plasma partly free and partly esterified or combined Differential precipitation with digitonin enables the two forms to be calculated by difference when measured colorimetrically in acetic anhydride and sulfuric acid

Apparatus

1 Filter paper quantitative—extracted with hot alcohol until completely free of sterols
2 Preserving jars either pint or quart with rubber gaskets
3 A dark cabinet containing a water bath A wooden packing box equipped with door or curtain The cabinet is fitted with a pan or tray about 4 inches deep The larger the water bath the easier it will be to control the temperature Provisions should be made for a thermometer and a funnel for the addition of water by drilling holes in the top of the box
4 Cuvettes 19 × 150 mm
5 Coleman Jr Spectrophotometer Model 6
6 A quantity of 15 ml round bottom centrifuge tubes
7 A 110 C oven
8 An incubator set at 7 C
9 A quantity of 5 ml volumetric flasks
10 A quantity of stirring rods
11 A steam bath

Reagents

1 Acetone alcohol mixture One volume of redistilled acetone is mixed with one volume of absolute alcohol
2 Ether peroxide free
3 Acetone ether mixture One volume of redistilled acetone to two volumes of peroxide free ether
4 Digitonin solution Dissolve 400 mg in 100 ml of distilled water by heating on a steam bath The solution *must be clear* A sediment will develop on standing but this can be removed by filtration or centrifuging before use
5 Potassium hydroxide solution 33% in water
6 Phenolphthalein solution a 1% solution in alcohol
7 Acetic acid solution Dilute 10 ml acetic acid up to 100 ml with distilled water
8 Glacial acetic acid (ACS or reagent grade)
9 Acetic anhydride 99 to 100% chloride free
10 Sulfuric acid concentrated
11 Stock cholesterol solution in glacial acetic acid 100 mg of cholesterol per 100 ml of glacial acetic acid The cholesterol must be pure white and odorless and it must melt sharply at not below 147 C If not recrystallize from an anhydrous solvent such as absolute alcohol methyl alcohol or ethylene chloride
12 Use a working standard of 0 1 mg/ml Dilute 10 ml of the stock cholesterol to 100 ml with glacial acetic acid
13 Acetic anhydride sulfuric acid mixture Add acetic anhydride (enough for a series of determination) into a glass stoppered flask chill it in an ice bath and add concentrated sulfuric acid in the proportion of 1 ml to 20 ml of anhydride agitate the contents during the addition while the flask is kept in the ice bath Insert the stopper remove from the bath shake vigorously for a few moments and return to the bath

Procedure

1 *Preparation of Sample*

a Pipette 7 to 10 ml of acetone alcohol solution into a 25 ml volumetric flask and pipette exactly 1 ml of serum into the solution Mix after the addition of the serum by swirling the solution

b Heat the flask over a steam bath and rotate the flask to prevent bumping

c When the solution boils cool the flask to room temperature and dilute to the mark with acetone alcohol solution

d Filter into a clean flask

e Pipette samples for free cholesterol and total cholesterol at once to avoid concentration changes due to evaporation

2 *Isolation of Free Cholesterol*

a Pipette 7 ml of the filtrate into a 15 ml centrifuge tube add approximately 3.5 ml of digitonin solution and one drop of 10% acetic acid solution

b Stir thoroughly and leave rod in the tube Place the tube in a preserving jar cover tightly and leave it overnight at room temperature

c Transfer tube to a rack tir to break up clumps of precipitate and to free particles which may adhere to the sides of the tube near the surface of the liquid Remove the rod and place it in the rack making certain that no precipitate on rod is lost

d Centrifuge until the precipitate is packed tightly enough to permit decanting without any loss Then drain the tube for a few moments by touching the lip to a clean towel

e Replace the stirring rod and wash down the wall of the tube and rod with approximately 4 ml of acetone ether solution

f Stir thoroughly return rod to rack centrifuge and decant again

g Wash the precipitate twice more with ether instead of the acetone ether mixture

h Replace the rod and set the tube aside for color development (Be sure that the ether has been completely evaporated before proceeding)

3 *Total Cholesterol Digitonide*

a Add 3 drops of the 33% KOH solution to a 15 ml centrifuge tube pipette 3 ml of the extract into the tube and stir at intervals with a vigorous up and down motion of a stirring rod until no droplets of the alkali can be se n at the tip of the tube

b Place a layer of sand about 5 cm deep in a preserving jar and heat it in a water bath until the temperature of the sand is about 4, C Place the tube in the sand cover the jar tightly and put it in an incubator 37 to 40 C for 30 minutes

c Remove the tube to a rack, allow to cool raise the stirring rod and add alcohol a etone to the 6 ml mark

d Then add 1 drop of phenolphthalein solution and titrate with 10% acetic acid solution About 0 4 ml should be required Add one drop in excess

e Add 3 ml of the digitonin solution stir thoroughly and place the tube in a preserving jar Cover it tightly and leave it at room temperature for at least *3 hours* *preferably* overnight Carry out the centrifuging and washing as described for free cholesterol except that in this case only one ether washing is necessary

4 *Development of Color in All Specimens*

a Heat a shallow pan containing a layer of sand (about 3 cm deep) to *110 to 115* in an oven *Place the tubes* containing the precipitate in the sand in the order in which readings are to be taken and return the pans to the oven for 30 minutes

b While the precipitates are drying in the oven adjust the *water bath to* 5 C and maintain it at this temperature during the rest of the procedure

c After 30 minutes remove the sand bath from the oven and pipette _ ml of glacial acetic acid into the first tube while it is still in the hot sand

d Allow the acid to wash down the rod and the wall of *the tube* Stir the contents vigorously and have the tube containing the rod in the sand while acid is being added to the next two or three tubes (2 or 3 minutes in all)

e Stir again remove the tube from the sand allow to cool and place it in a rack in the water bath Continue in this manner until acet c acid has been added to all the centrifuge tubes in the series to be read and all have been placed in the water bath

f In each series of determinations run at least two standards Pipette ␣ ml of standard cholesterol (0 1 mg per ml) into centrifuge tubes insert stirring rods and place in the water bath along with the unknowns Place one at the beginn ng and one at the end of the series of unknowns

g Start a stopwatch Then p pette 2 ml of glac al acetic acid and 4 ml of the mixed reagent into a cuvette mix thoroughly and use as a blank

h After 9 minutes remove one of the standards from the wate bath wipe it dry and pipette 4 ml of the mixed reagent into it

i Stir vigorously for a few moments and pour into a cuvette Leave in water bath for 3 minutes

j Then add 4 ml of mixed reagent to all the tubes shake vigorously and note time Pour into a cuvette and leave in a bath for 3 minutes

k Set the blank at 100% T (or D = 0) at 6 5 mμ Then remove each tube from the bath d y with a clean d y towel and read (be careful not to stir up the small quantity of sed ment at the bottom of the tube)

Calculation

mg % total cholesterol $= C_T = \frac{166\,7}{D} \times Du$

mg % free cholesterol $= C = \frac{71\,4}{D} \times Du$

mg % combined cholesterol $= C = C_T - C$

Per cent cholesterol combined $= \frac{100\ C}{C_T}$

Where Ds is the mean optical density of the standards and Du s the density of the unknown

Precautions

1 The centr fuging for free should be for 15 minutes at 800 r p m at the first stage For total 5 to 7 minutes is usually enough for the acetone ether and ether washings

The acetic anhydride reagent should be made up for each series of determinations Some heat is produced when mixed and a period of 10 minutes should be allowed before the reagent is used as an equil brium i reach d at that time

3 The wath bath should be mainta ned at ␣ C

4 Be careful of wate as it interferes with the reaction (avo d splashing)

4 Lipid Phosphorus in Blood and Serum

Reference

Youngsburg G E and Youngsburg M V Pho phorus Metabolism I A System of Blood Phosphorus Analysis J Lab & Clin. Med 16 158 166 (Nov) 1930

Principle

The extracted lip ds are oxidized with sulfuric acid and hydrogen peroxide and the phosphate present is measured by the method of Fiske and Subbarow

Apparatus

1 Large test tubes graduated at 0 ml.
2 A boiling water bath
3 A quantity of filter paper (quantitative)
4 A quantity of broken s l ca ware
5 Wire racks

6 An *electric hot plate or microburners*
7 A quantity of 25 ml volumetric flasks
8 Other apparatus as in phosphorus method (Fiske and Subbarow)

Reagents

1 Alcohol ether mixture Mix 3 volumes of 95% redistilled alcohol and one volume of redistilled ether
2 Sulfuric acid 5 N Dilute 50 ml of concentrated sulfuric acid to 360 ml with distilled water
3 Hydrogen peroxide 30% (Superoxol)
4 Ammonium molybdate a 2 5 per cent solution
5 Aminonaphtholsulfonic acid solution (ANSA) (see phosphorus method)

Procedure

1 Into a graduated 20 ml test tube pipette 18 ml of the alcohol ether mixture
2 Add exactly 1 ml of plasma or serum drop by drop
3 Mix and place in a boiling water bath until the contents of the tube boil Allow *to cool at room temperature*
4 Dilute to 20 ml with alcohol ether mixture mix and filter through quantitative filter paper
5 Pipette exactly 8 ml of the filtrate into a large test tube Add a silica pebble and place in a *wire rack*
6 Heat the rack and tubes over an electric hot plate until evaporated to dryness
7 Then add 2 5 ml of 5 N sulfuric acid and digest over a hot plate or microburner (Place the tube in a slanting position and heat until the mixture turns brown or black)
8 Allow to cool and add 1 drop of hydrogen peroxide allowing it to fall directly into the mixture
9 Replace the tube and continue heating until colorless
10 When colorless cool the tube add some water heat again and transfer the contents to a 25 ml volumetric flask washing with water to about 15 ml volume
11 Then add 2 5 ml of ammonium molybdate solution
12 Add 1 ml of ANSA reagent mix and dilute to 25 ml with water
13 For a blank add 2 ml of water and digest with sulfuric acid and peroxide as above in Steps 7 10
14 Using the Fiske and Subbarow phosphorus curve set the blank at 100% T at 660 mμ and read the unknowns

Calculation

1 Use the phosphorus curve of the method of Fiske and Subbarow
2 mg lipid phosphorus (as P)/100 ml serum =

$$\frac{(\text{mg P/ml unknown}) \times (\text{dilution 1}) \times (\text{dilution 2}) \quad 100}{(\text{aliquot 1}) \times (\text{ml serum})}$$

Example

A reading of 85% T was equivalent to a concentration of 0 0015 mgP/ml in the cuvette In the analysis one ml of serum was extracted in 20 ml of alcohol ether of which 8 ml were finally diluted to 25 ml in the phosphorus analysis

mg lipid phosphorus (as P)/100 ml serum =

$$\frac{(0\ 0015) \times 20 \times 25 \times 100}{8 \times 1} = 9\ 4 \text{ mg P/100 ml}$$

Precautions

1 These in general are the same as for phosphorus estimations

5 Acetone Bodies

A. TOTAL IN BLOOD

References

Van Slyke D D Studies of Acidosis VII J Biol Chem 32 455 494 (Dec) 191

Van Slyke D D and Fitz P Stud es in Acidosis VIII The Determination of β Hydroxybutyric Acid Aceto acetic Ac d and Acetone in Blood J B ol Chem 32 49 497 (Dec) 1917

Principle

Acetone in hot sulfuric acid olutions forms an insoluble precipitate w th mercuric sulfate Aceto acetic acid breaks down to acetone on heating and beta hydroxy butyric acid treated with potass um dichromate in hot ac d solutions forms acetone The three compounds may the efore be estimated by weigh ng the mercuric acetone sulfate

Apparatus

1 Reflux condensers with interchangeable 500 ml Erlenmeyer flasks
An oven set at 110 C
3 S ntered glass Pyrex crucibles with medium po os ty
4 Volumetr c p pettes 8 ml or an accurately calibrated syringe p pette
5 A desiccator
6 An accu ate analytical balance
7 Quant tative filter pape
8 Graduated cylinders 50 ml capacity
9 Volumetric flasks 100 ml

Reagents

1 Copper sulfate ($CuSO$ $5H O$) a 10% solution in water
Calcium hydroxide ($CaOH$) a 10% suspension in water
3 Mercuric sulfate ($HgSO$) Dissolve 73 gm of red oxide of me cury n 1000 ml of 4 N sulfuric acid Gentle warming may be requi ed
4 Sulfuric acid Make a 4 N solut on by diluting 40 ml of concentrated $H SO$ to 60 ml of water
5 Potassium d chromate ($K Cr O$) a 5% solution in wate
6 Sulfur c acid ($H SO$) 17 N 1/0 ml of concentrated sulfuric acid d luted to 360 ml of water
7 A mixture of $HgSO$ solution and 17 N sulfuric ac d in the ratio 35 volumes $HgSO$ to 10 volumes $H SO$
8 Sod um hydroxide 5 N 0 gm of sod um hydroxide made up to 100 ml of water

Procedure

1 *Preparat on of Blood*

a Pipette exactly 8 ml of blood into 80 ml of water in 00 or 50 ml graduated cylinders Mix
b Add with mixing 16 ml of 10% HgSO Allow to stand one-half hour or more
c Dilute to 00 ml with distilled water Mix and allow to stand 1 hour or over n ght in refrigerator
d Filter through quant tat ve filter paper
e Use 100 125 ml of filtrate (= 5 ml blood) for analysis

Preparation of Sintered Glass Filter

a Add 10 15 ml of aqua regia to cup and let stand overnight Filter and wash out by running 00 ml of hot distilled water through filter Dry 1 hour at 110 C Cool for one half hou in des ccator and we gh

5 *Precipitation of Mercuric Acetone Sulfate*

a To a wide mouthed flask add 45 ml of $HgSO_4 - H_2SO_4$ reagent
b Add 125 ml of blood filtrate
c Attach to a reflux condenser
d Light a medium sized flame
e Bring to a boil
f Add 5 ml of potassium dichromate solution If the dichromate is added before solution comes to a boil the oxidation is to acetic acid instead of to acetone so that there is a significant loss
g Boil for 90 minutes after adding dichromate Keep boiling until just before filtering them through Pyrex crucibles
h Filter while still very hot using suction Wash with 100 200 ml of water
i Dry one hour at 110 C Weigh after cooling for one half hour

Calculation

1 In this method certain assumptions have to be made in the calculation
a That 1 mg acetone yields 20 0 mg precipitate, that 1 mg aceto acetic acid yields 11 4 mg precipitate and that both substances react quantitatively
b That beta hydroxybutyric acid yields only 75% under the conditions of this method and that 1 mg of beta hydroxybutyric acid yields 8 45 mg of precipitate
c That in most specimens of blood and urine the molecular proportion of beta hydroxybutyric acid is 75% of the total acetone bodies
d. Under the conditions described the following factors are valid when the equivalent of 5 ml of blood is analyzed and calculated as acetone For total acetone bodies 1 mg of precipitate equals 1 28 mg of total acetone bodies/100 ml blood for beta hydroxybutyric acid 1 mg of precipitate equals 1 3? and for acetone + aceto acetic acid, 1 mg of precipitate equals 1 00

2 mg total acetone bodies (as acetone)/100 ml blood = (mg ppt) × 1 28

Example

The precipitate from the blood of a diabetic weighed 1 1 mg

mg total acetone bodies (as acetone)/100 ml blood = 1? 1 × 1 28 = 15 5 mg/100 ml

Precautions

This method is rather empirical Hence great care must be taken to follow the instructions explicitly

B TOTAL IN URINE

Procedure

1 *Preparation of Urine*

a Pipette exactly 10 ml of urine into 100 ml volumetric flask Add about 30 ml water
b Add 20 ml copper sulfate reagent
c If not turbid add approximately 5 N NaOH drop by drop until turbidity persists
d Add 20 ml calcium hydroxide reagent
e Dilute to mark shake and keep in refrigerator at least one hour preferably over night
f Filter

2 *Blank for Urine*

a Add 25 ml filtrate to a 125 ml flask
b Add 0 5 ml 17 N sulfuric acid
c Boil not less than 5 nor more than 10 minutes
d Filter and wash with 100 ml of water into 45 ml of mixed reagent ($HgSO_4 - H_2SO_4$)

e Bo l in condenser not more than 45 minutes Do not add dichromate
f Remove and filter through sintered glass filter
g We gh

3 *Prec p tat on of Mercur c Acetone Sulfate*
a To a 500 ml flask add 45 ml of HgSO H SO reagent 25 ml of urine filtrate and 100 ml water
b Pro eed as under blood

Calculations

1 For urine the same cons derat ons hold as for blood For ur ne under the cond t ons of this method (e the equ valent of 5 ml urine analyzed) the factors are total a etone bod es 1 mg ppt — 2 56 for beta hydroxybutyr c ac d 2 64 and for acetone 00
mg total acetone bod es (as ac tone)/100 ml urine — (mg ppt mg blank) × 2 56

Example

The ur ne from a mild d abet c was analyzed The blank we ghed 1 mg the un kno vn 5 6

mg total acetone bod es (as acetone)/100 ml urine = (5 6 − 1 2) × 2 56 = 11 2 mg/100 ml

Precautions

Same as for blood

C INDIVIDUAL ACETONE BODIES

Principle

Acetone reacts d rectly w th mercur c sulfate n hot ac d solut on aceto acet c ac d breaks down quant tat vely to carbon d ox de and acetone wh ch reacts at once beta hydroxybutyric ac d yields acetone only when an ox diz ng agent (d chromate) is present

Apparatus and Reagents

Same as for blood total acetone bod es

Procedure

1 In e ther blood or urine determ ne acetone plus aceto acet c ac d by running through the procedures descr bed above but w th the following alterat ons
a Do not add d chromate
b Bo l for not less than 30 nor more than 45 m nutes
In e ther blood or ur ne determ ne beta hydroxybutyr c ac d by runn ng through the whole procedure nclud ng d chromate after the follow ng prel minary steps to remove acetone and aceto acet c ac d
a The filtrates equ valent to 5 ml blood or 2 5 ml ur ne are m xed w th 100 ml water and 2 ml 50% sulfur c ac d.
b They are bo led 10 m nutes n an open flask and cooled
c They are d luted to 1 5 ml w th water

Calculation

1 mg (acetone plus aceto acet c ac d as acetone)/100 ml = mg ppt × 1 00 (for blood)
(mg ppt mg blank) × 2 00 (for ur ne)
2 mg beta hydroxybutyr c ac d (as acetone)/100 ml = mg ppt × 1 32 (for blood)
(mg ppt mg blank) × 64 (for ur ne)

6 Fats and Derivatives, Miscellaneous References

Acetoacetic Acid

Rosenthal S M A Colorimetric Method for the Estimation of Acetoacetic Acid in the Blood J Biol Chem 179 1 35 1?44 (July) 1949

Acetone

Adams C A and Nicholls J R The *Analysis of Mixtures Containing Acetone Ethyl* Alcohol and Isopropyl Alcohol, Analyst 54 2 9 (Jan) 19 9

Behre J A A Modified Salicyaldehyde Method for the Determination of Acetone Bodies in Blood and Urine J Biol Chem 136 25 34 (Oct) 1940

Greenberg L A and Lester D A Micro Method for the Determination of Acetone and Ketone Bodies J Biol Chem 154 (1) 177 190 (June) 1944

Huckabay W B Newton C J and Mettler A V Optimum Conditions for Titrimetric Determinations Determination of Acetone, Anal Chem 19 838 841 (Nov) 1947

Rappaport, F and Baner B Microdetermination of Acetone and Diacetic Acid in Blood J Lab & Clin Med 28 1770 1772 (Nov) 1943

Rothera A C H Note on the Sodium Nitroprusside Reaction for Acetone J Physiol 37 491 494 (Dec) 1908

Acetyl Acetone

Witter R F Snyder J and Stotz E Colorimetric Determination of Acetylacetone and Related β Diketones J Biol Chem 176 493 500 (Nov) 1948

Cholesterol

Kingsley, G R and Schaffert R R Determination of Free and Total Cholesterol by Direct Chloroform Extraction J Biol Chem 180 (1) 315 3?3 (Aug) 1949

Fatty Acids

Bloor W R A Colorimetric Procedure for the Determination of Small Amounts of Fatty Acid J Biol Chem 170 671 674 (Oct) 1947

Formic Acid

Bastrup J T Method for the Determination of Formic Acid in Urine Acta Pharmacol et Toxicol 3 303 311 1947

Grant W M Colorimetric Micromethod for Determination of Formic Acid Anal Chem *19 (3) 206 207 (March) 1947*

Hydroxybutyric Acid

Shaffer P A and Marriot W M The Determination of Oxybutyric Acid J Biol Chem 16 265 80 (Sept Oct) 1913

Phospholipid

Egsgaard, J Determination of the Phosphatide Content of Serum Acta Physiol Scandinav 16 (/3) 171 178 1948

Hack M H Estimation of the Phospholipides in Human Blood J Biol Chem 169 (1) 137 143 (June) 1947

Harnes A R The Colorimetric Determination of Lipoid Phosphorus in Blood J Biol Chem 77 405 408 (May) 19 8

Total Fat

Bauer F C Jr, and Hirsch E F Esterified Fatty Acid Levels of Normal Human Sera Arch Biochem 23 (1) 137 140 (Aug) 1949

Fowweather F S and Anderson W N A Method for the Determination of Fat in *Feces* Biochem J 40 (3) 350 351 1946

Stoddard J L and Drury I E A Titration Method for Blood Fat J Biol Chem 84 741 748 (Nov) 19 9

van de Kamer J H ten Bokkel Huinink H and Weyers H A Rapid Method for the Determination of Fat in Feces J Biol Chem 177 347 355 (Jan) 1949

Zuckerman J L Zymaris M C and Natelson S The Simple Method for the *Determination of* Fecal Fat and Fatty Acids J Lab & Clin Med *34* ?8- ?86 *(Feb)* 1949

SECTION IV

BIOCHEMICAL PROCEDURES (Continued)

E VITAMINS

1 Vitamin A and Carotenoids (Carr Price)

A. IN BLOOD AND SERUM

Reference

May C D Blackfan K D McCreary J F and Allen F H Jr Clinical Studies of Vitamin A in Infants and Children Am J Dis Child 59 1167 1184 (June) 1940

Principle

Materials containing vitamin A when treated by a chloroform solution of antimony trichloride form a blue color (Carr Price reaction) Experience has shown a definite association between the blue color and vitamin A content of a wide variety of materials as determined by biologic assay

Apparatus

1 A special container for the antimony trichloride This reagent has a tendency to pick up water easily and as a result the blue color is impossible to read in the colorimeter
2 Cuvettes—Coleman 10 x 75 mm
3 Air pressure to evaporate the ether
4 Centrifuge tubes—15 ml round bottom
5 An electric centrifuge
6 Coleman Jr Spectrophotometer Model 6 with the smallest cuvette holder (#6 108)
7 A water bath set at 45 C
8 Syringe pipettes 1 and 2 ml

Reagents

1 Antimony trichloride % in chloroform
2 Ethyl alcohol 95%
3 Petroleum benzine (ether) USP
4 Vitamin A alcohol standard Made up in petroleum ether one gamma per ml
5 Beta carotene standard 3 gamma/ml in petroleum ether (1 gm vitamin A alcohol is 4 500 000 International Units i e 1 IU is 0 2 micrograms 1 I U is 0 6 micrograms beta carotene)

Procedure

1 To exactly 1 ml of serum add 2 ml of 95% ethyl alcohol
2 Then add ml of petroleum ether stopper tightly and shake vigorously and continuously for 10 minutes for complete extraction

3 Centrifuge and pipette out accurately 1 ml of the ether layer into a small cuvette Read immediately at 440 mμ the petroleum ether blank being set at 100% T

4 The yellow color of the ether layer is due to the carotenoid pigments the bile pigments being unextracted under these conditions The principal carotenoids in blood serum are carotene and xanthophyll Estimation of the total amount of carotenoids is necessary in the determination of vitamin A, in order that the measurement of the total blue color formed by the Carr Price reaction may be corrected for that portion arising from the carotenoids

5 Evaporate ether at 40 to 45 C to dryness by gently blowing dry air into the cuvette while it is partially immersed in water Make sure that the ether odor is completely gone and that the cuvette is perfectly dry Otherwise the Carr Price color will be a cloudy blue

6 Place an antimony trichloride blank at 100% T at 620 mμ

7 Add exactly 1 ml of antimony trichloride to the dry cuvette and read immediately The galvanometer reading is taken at the point of maximum absorption of light that is maximum color formation This is necessary as the blue color fades very fast (20 40 seconds)

Calculation

1 Run a calibration curve for carotenoids using beta carotene in the range 0 to 4 mcg/ml at 440 mμ

2 Run a calibration curve for vitamin A alcohol in the range 0 to 1 5 mcg/ml at 620 mμ and following all the steps in the procedure

3 $$\text{mcg carotenoids/100 ml serum} = \frac{(\text{mcg in cuvette at 440 m}\mu) \times (\text{dilution}) \times 100}{(\text{ml aliquot}) \times (\text{ml serum})}$$

4 mcg vitamin A alcohol/100 ml serum =

$$\frac{(\text{mcg in cuvette at 620 m}\mu) \times (\text{dilution}) \times 100}{(\text{ml aliquot}) \times (\text{ml serum})} - (0\ 11 \times \text{mcg carotenoids})$$

5 The correction factor for blue color due to carotenoids is necessary in Equation 4 and was established empirically for a mixture of carotene and xanthophyll in the proportions usually present in serum

Example

At 440 mμ the reading was 85 0% T equivalent to 0 70 mcg beta carotene in the cuvette

At 620 mμ the reading was 80 5% T equivalent to 0 40 mcg in the cuvette One ml of serum was extracted into 2 ml of petroleum ether of which 1 ml was used for analysis

1 $$\text{mcg carotenoids/100 ml serum} = \frac{(0\ 70) \times (2)\ (100)}{(1) \times (1)} = 140 \text{ mcg/100 ml}$$

2 $$\text{mcg vitamin A/100 ml} = \frac{(0\ 40) \times (2) \times (100)}{(1) \times (1)} - (0\ 11 \times 140)$$

$$= 80 - 15 = 65 \text{ mcg/100 ml}$$

Precautions

1 Be sure the antimony trichloride contains no water otherwise the solution will be cloudy

2 Be sure the sample is dry before the addition of antimony trichloride

B VITAMIN A IN FECES

Reference

Friedemann T I ersonal communication

Apparatus

1 Water bath set at 60 C
2 Electric centrifuge etc as for serum
3 An analytical balan e
4 A quantity of 100 ml volumetric flasks
5 A quantity of 100 ml graduated cyl nders

Reagents

1 Phosphoric ac d (syrupy)
2 Sodium sulfate (anhydrous)
3 Sodium hydroxide (NaOH) 10 N 40 gm/100 ml water
4 Caprylic alcohol
5 Ethyl alcohol 50% in water
6 Methyl alcohol 70% in water
7 Oth r reagents are the same as for serum vitamin A

Procedure

1 Weigh out 10 gm of mixed feces into a 100 ml volumetric flask
2 Add 50 ml of 95% alcohol 5 ml of 10 N NaOH and 1 ml of caprylic alcohol
3 Heat for 0 minutes at 60 C cool and dilute to 100 ml
4 Pipette 10 ml of diluted feces into a 100 ml cylinder
5 Add 10 ml of water and 0 ml of petroleum ether
6 Shake for 5 minutes and remove top layer to a new test tube
7 Add 5 ml of syrupy phosphoric acid and shake well (The phosphoric acid takes away interfering material without removing any of the vitamin A)
8 Remove phosphoric acid layer and wash with 10 ml of 50% ethyl alcohol
9 Dry out water with sodium sulfate and decant solution to measure color
10 To remove carotene shake two times with 70% methyl alcohol This step will take out the carotene and also a little of the vitamin A
11 Cont nue as n method for serum vitamin A

Calculation and Precautions

See vitamin A in s rum

2 Vitamin A and Carotenoids (Dichlorohydrin)

Reference

Sobel A E and Snow S D The Est mation of Serum Vitamin A With Activated Glycerol Dichlorohydrin J Biol Chem 171 617 63? (Dec) 194

A. USING THE COLEMAN UNIVERSAL MODEL 11

Principle

A solut on of activated glyce ol dichlorohydrin (GDH) when added to a chloroform solution conta ning vitamin A produces a violet color Advantages over the anti mony trichloride method are principally two first the color is stable for two to 10 minutes second the reagent is not affected by traces of mo sture The inter ference of vitam ns D and D ergosterol 7 dehydrocholesterol and cholesterol with the GDH reaction has been found to be negl gible

Apparatus

1 It is n ce sary to measure s mples at 800 mμ or above Hence the Coleman Uni versal Spectrophotometer Model 11 is recommended

2 1 3 cm cuvettes and cuvette carrier obtained from the Coleman Electric Company
3 ✓0 cm cuvettes obtained from the Pyrocell Manufacturing Company 07 East 84th Street New York 28 Carrier obtained from the Coleman Electric Company
4 555 mμ filter (filter combination 348° M608 1 0 mm and 5300, M954 4 0 mm) obtained from the Corning Glass Works Corning New York
5 An electric oven set at 60 C
6 A water bath set between 40 50 C
7 An electric centrifuge
8 Syringe pipette 1 ml accurately calibrated
9 A quantity of flat tipped glass stirring rods

Reagents

1 95 percent ethanol (USP)
2 1 N KOH in approximately 90 percent ethanol freshly prepared 5 6 gm KOH/100 ml
3 Petroleum ether b p 30 60 C
4 Nitrogen Ohio Chemical Company
5 Sodium sulfate (Na_2SO) anhydrous analytical reagent grade
6 Chloroform Analytical reagent grade is washed with water distilled the first and last fractions are discarded and the remainder is kept over dry Na_2SO_4.
7 Antimony trichloride, analytical reagent grade
8 Carotene 90 percent beta 10 percent alpha, from General Biochemicals Inc
9 Standard vitamin A solution About 40 to 50 mg of a vitamin A concentrate which comes in gelatin capsules control PC 3 from Distillation Products Inc were diluted in chloroform to give the desired concentration of vitamin A The concentrate has $E\ \frac{1\%}{1\ cm}$ value in absolute ethanol of 100 75 This value was supplied by the manufacturers and agreed with that found by the authors Multiplying this value by 2000 the standard commercial conversion factor permits estimation of the vitamin A potency at 2 015 000 USP units per gm If the extinction of the concentrate is divided by the extinction of crystalline vitamin A alcohol in ethanol 1780 the vitamin A content of the concentrate becomes by simple proportion 5 56 percent
10 Activated GDH Glycerol dichlorohydrin (Eastman Kodak practical grade a mixture of 1 3 and 2 3 dichlorohydrin from the Shell Chemical Company) is vacuum distilled in the presence of approximately 1 percent by weight of SbCl at from 10 to 40 mm of pressure the first and last fractions being discarded. The distilled reagent should be colorless and clear (a sample of the reagent should not become even slightly cloudy on the addition of water) The presence of SbCl in the distillate (shown when a sample of GDH becomes cloudy upon the addition of water) can be remedied by a second vacuum distillation The activated reagent may be obtained from the J B Shohan Laboratories 78 Wheeler Point Road Newark 5 New Jersey

Procedure

1 Exactly 1 ml of serum is pipetted into a ⅝ × 4 inch test tube
2 Add 1 ml of 95 percent ethanol and mix by tapping the tube (or for saponification 1 ml of the 1 N KOH in 90 percent ethanol is added and the contents of the tube mixed and placed in a 60 C oven for 20 minutes)
3 Add 2 ml of analytical reagent p troleum ether and shake for 10 minutes
4 After shaking the tube is centrifuged for about 30 seconds
5 The supernatant petroleum ether is aspirated and placed in a ½ × 4 inch test tube The aspirator is a fine tipped dropper

6 With another 9 ml of the petroleum ether and shaking for only five minutes the extraction procedure is repeated
7 The extract is evaporated to dryness by placing the tube in a 40 50 C water bath and running a stream of nitrogen over it
8 Add 1 ml of analytical reagent grade chloroform to bring the dried extract into a solution
9 Then add 4 ml of GDH and m x with a flat tipped stirring rod.
10 Two m nutes after m xture the solution is placed in a ‿0 mm cuvette The ab sorption of the solution is measured first at 550 mμ (with the 555 mμ filter) against a blank cons sting of 4 ml of GDH and 1 ml of chloroform When the concentration of vitam n A is h gh read at 4 m nutes
11 The wave length dial is then turned to 800 mμ the 555 mμ filter replaced with filter PC 5 and at 4 minutes after the initial mixture of the reagents the absorp t on is read at 800 mμ

Calculation

1 Prepare the calibrat on curve for vitam n A as follows
   a Prepare solutions of vitamin A in chloroform solut on containing standard solution as well as the GDH preheated to 25 C before use
   b To 1 ml of the standard in a glass stoppered cylinder are added 4 ml of GDH
   c The contents of the cyl nder are mixed by inversion and are placed in a 25 C water bath for minutes The solution is then poured into the 5 cm cuvette
   d The absorption of the p nk color produced is read against a blank consisting of 4 ml of GDH and 1 ml of chloroform The instrument is used with the wave length scale set at 550 mμ in conjunction with a 555 mμ filter

Prepare the cal bration curve for carotene at 800 mμ as follows
   a Prepare standards of carotene in chloroform in the range 0 10 mcg/ml
   b Carry these through all steps of the procedure reading them against a chloro form blank at 800 mμ us n filter PC 5

3 Prepar a curve for correcting for carotene interference at 550 mμ as follows
   a Prepare carotene standards in chloroform in the range 0 10 mcg/ml.
   b Run these through the vitamin A method reading them against the blank set at 100% T at 50 mμ with the 555 mμ filter
   c Determ ne tl e interference factor of carotene at 550 mμ i e the apparent vitamin A n mcg due to 1 mcg of carotene/100 ml serum when measured at 550 mμ

4 $\text{mcg carotene/100 ml serum} = \dfrac{(\text{mcg in cuvette at 800 m}\mu) \times 100}{(\text{ml serum})}$

5 mcg vitamin A/100 ml serum =

$$\frac{(\text{mcg in cuvette at 550 m}\mu) \times 100}{(\text{ml serum})} - (\text{Factor} \times \text{mcg ca otene/100 ml serum})$$

Example

1 At 800 mμ the read ng was 78% T equivalent to 1 6 mcg carotene in the cuvette At 550 mμ the reading was 81% T equivalent to 1 9 mcg v tam n A in the cuvette One ml of serum had been extracted into 4 ml of petroleum ether all of which was evaporated to dryne s and treated in the cuvette with 1 ml chlo oform and 4 ml of GDH

2 $\text{mcg carotene/100 ml serum} = \dfrac{(1\ 6) \times 100}{1} = 160 \text{ mcg/100 ml}$

3 mcg vitamin A/100 ml serum =

$$\frac{(1\ \ ) \times 100}{1} - (0\ 15 \times 160) = (1\ 0 - {}^{n}4) = 96 \text{ mcg/100 ml}$$

Precautions

1 In this method exact timing *is necessary for good results*
2 When the concentration of vitamin A is high read at 4 minutes instead of 2

B VITAMIN A (DICHLOROHYDRIN METHOD) WITH BECKMAN SPECTROPHOTOMETER

Apparatus

1 A Beckman Spectrophotometer Model DU
2 Four matched Corex cells with 10 mm light path absorption cells
3 A four place cell holder
4 Syringe pipettes 0 6 ml and 2 4 ml accurately calibrated
5 Other apparatus same as for Coleman Universal Model 11

Procedure

1 Same as procedure for Coleman Universal up to and including step 7
2 Add 0 6 ml of chloroform with a syringe pipette
3 Stir and add 2 4 ml GDH
4 Read at 2 minutes but *if the sample is high (a tolerance test) read between 3* and 4 minutes
5 Read at 555 mμ setting the blank at 100% T
6 Change wave length to 830 mμ and read again setting the blank at 100% T

Calculation

1 The calculation and calibration is the same as for the Coleman Model 11

Precaution

1 Use glass stoppered tubes for extraction
2 Saponification is a critical step complete saponification is essential

3 Thiamine in Urine Food Blood and Feces

A. IN URINE

Reference

Hennessey D J and Cerecedo L R The Determination of Free and Phosphorylated Thiamin by a Modified Thiochrome Assay J Am Chem Soc 61 179 183 (Jan) 1939
Connor R T and Straub G J Combined Determination of Riboflavin and Thiamine in Food Products Ind and Eng Chem Anal Ed 13 385 389 (June) 1941

Principle

Thiamine is adsorbed from urine in acid solution by means of activated zeolite It is eluted with concentrated potassium chloride and is converted to thiochrome by ferricyanide in alkaline solution Thiochrome is extracted into isobutyl alcohol and measured fluorometrically

Apparatus

1 Photofluorometer Coleman Model 12 or 12B
2 Hennessey adsorption tubes or columns with long (5 to 5 5 inch) stems
3 Fine glass wool
4 Reaction vessels 25 ml glass stoppered (interchangeable stoppers) vessels with a conical bottom
5 Reading cuvettes Coleman 19 × 150 mm
6 Syringe pipettes—5ml *10 ml*
7 Wooden applicators for inserting the glass wool into the columns

Reagents

1 Potassium chloride 25% solution Dissolve ~0 gm KCl in 1500 ml 0 1 N HCl with the aid of heat Filter
Bromcresol green indicator 0 4% in 70% alcohol.
3 Sodium acetate 2 5 M Dissolve 330 gm in 1000 ml distilled water
4 *Decalso* Wash 60 to 80 mesh Decalso 3 times with 3% acetic acid once with 25% KCl again with 3% acetic acid and then several times with distilled water Each washing consists of stirring the Decalso 15 minutes in the wash settling and decanting
5 Sodium hydroxide 1 N solution Dissolve 40 gm of sodium hydroxide in 1000 ml of distilled water
6 Sodium sulfate anhydrous
7 Acetic acid 0 5% solution in water
8 Isobutyl alcohol redistilled
9 Potassium ferricyanide a 1% solution in water This solution is stable for 6 months if kept in a dark bottle
10 Potassium hydroxide a 15% solution in water
11 Oxidizing reagent One part of 1% ferricyanide solution is mixed with 9 parts of potassium hydroxide (15%) Prepare fresh every day
12 Quinine sulfate standard
Stock standard 100 mg USP quinine sulfate per liter of 0 1 N H_2SO_4 Stable for 1 year
Intermediate standard 25 ml stock standard dilute to 100 ml with 0 1 N H_2SO_4
Working standard 5 ml intermediate standard to 500 ml 0 1 N H_2SO_4. Prepare daily
13 Thiamine standard (100 mg/ml) Dissolve 50 mg USP thiamine standard in 200 ml of 95% C_2H_5OH Add 5 ml 0 1 N HCl and bring to 500 ml with distilled water Store in refrigerator It is stable for 6 months.

Collection of Sample

Individual samples are preserved by adding 0 5 ml of 10 N H_2SO_4 to each 100 ml of sample 24 hour samples are collected in amber colored 1 gallon jugs which contain 5 ml of 10 N H_2SO_4 The vitamin content of the acidified sample will remain unchanged over several weeks if the sample is kept in the refrigerator A suitable aliquot is transferred to a 150 ml extraction flask the reaction is adjusted to pH 3 5 and the adsorption is carried out as described below

Procedure

1 Place a small piece of glass wool in the tip of the micro adsorption column, fill the column with activated Decalso so that the bottom of the bulb is just covered
2 Wash the column with 3 ml 0 5% acetic acid and allow to drain
3 Take a 5 ml aliquot of urine in a 50 ml Erlenmeyer flask add 2 drops of bromcresol green and neutralize to a light greenish yellow color with 1 N NaOH
4 A blank consisting of 5 ml 0 5% acetic acid is run through the method with each set of samples. Run the samples through the columns and allow to drain by gravity into a waste pan
5 Rinse the flasks with 3 ml 0 5% acetic acid and put this through the columns
6 After this has drained through the columns are completely drained by using a small amount of air pressure at the top of the column Recoveries of thiamine standard added to urine should also be run along with each batch of unknowns
7 Reaction vessels are placed under the adsorption columns and the columns are eluted with 5 ml 25% KCl. After this has drained the columns are again blown out with a small amount of air pressure
8 To the 5 ml eluate 0 7 ml of oxidizing reagent is added with mixing

9 Then add 10 ml isobutanol stopper with the gla s stopper and shake 50 times. Each sample is done individually and after the layers have settled the bottom layer is drawn off with suction.

10 A small amount of anhydrous Na_2SO_4 is added and the vessel is agitated gently The isobutanol layer must be clear after the sulfate has settled. If not repeat this step

11 The sample is then decanted into cuvettes to be read on the Coleman Model 1[2] Photofluorometer

12 The B filters are used and after the machine has warmed up and has been balanced the working quinine sulfate standard is used to set the machine at 60

Preparation of Standard Curve

1 Dilute the stock thiamine solution to 1 mcg/ml in 0 1 N H_2SO_4 to which has been added 10 ml 2.5 M sodium acetate/100 ml Run a series of standards in the range 0 2 mcg per tube following all steps of the procedure

2 Plot these values as described in the section on fluorometry

Calculation

1 $$\text{mcg thiamine/100 ml urine} = \frac{(\text{mcg in cuvette} - \text{blank}) \times (\text{total ml eluate}) \times 100}{(\text{ml eluate}) \times (\text{ml urine})}$$

2 The problem of the blank has never been solved completely in this procedure because urine contains many interfering substances In particular N methyl nicotinamide is adsorbed and eluted like thiamine fluoresces in alkaline solution and is only partially destroyed by ferricyanide Najjar has recommended a blank consisting of urine treated in every way as for thiamine omitting the ferri cyanide step and making an empirical correction Mickelsen has recommended washing the final isobutyl layer with acid to remove non thiochrome fluorescence The reader will have to make his own choice of blank.

Example

The unknown samples read an average of 55 and the blank 5 equivalent to 0 68 mcg and 0 06 mcg of thiamine respectively in the cuvette Five ml of urine had been used and eluted with 5 ml of KCl all of which was oxidized

$$\text{mcg thiamine/100 ml urine} = \frac{(0\ 68 - 0\ 06) \times 5 \times 100}{5 \times 5} = 12\ 4\ \text{mcg/100 ml}$$

Precautions

1 The samples must be shaken immediately after the addition of the oxidizing solution

2 Be sure the Decalso is activated before using it

3 The isobutyl alcohol must have a low blank if it does not redistill it until it does read below 5 on the scale

B THIAMINE IN FOOD BLOOD AND FECES

Reference

Same as for thiamine in urine

Principle

Same as for thiamine in urine except that in food blood and feces bound thiamine has to be hydrolyzed to liberate free thiamine

Apparatus

1 Digestion tubes The same tubes are used for digestion as for nonprotein nitrogen. They are sufficiently accurate for many types of samples to serve as receiving tubes for the eluate Heavy walled tubes made from ignition

tubes should be specified. It is necessary that they be heavy walled since they are also used as centrifuge tubes in some analyses (blood). As purchased they are calibrated at 1 5 25 35 and 50 ml

2 J shaped stirring rods These are made from 1 inch lengths of 3 mm glass rod. The round bend at one end fits the bottom of the tube With such a rod the contents of the tube can be violently agitated by rolling the rod back and forth between the thumb and index finger

3 Hennessy adsorption tubes with the long (5 to 5.5 inch) stems are recommended.

4 Reaction vessels Centrifuge tubes of about 50 ml capacity which are glass stoppered and pointed at the bottom (Wilkens Anderson #7105 GMK)

5 Waring Blendor

6 Coleman Photofluo ometer

7 *Cuvettes 19 × 150 mm*

8 An incubator set at 45 C

Reagents

These are the same as for thiamine in urine with a few additions

1 Hydrochloric acid 0.1 N Mix 1 ml of conc HCl with 110 ml of water

Sodium bicarbonate To prepare a molar solution dissolve 84 gm up to 1000 ml with water

3 *Metaphosphoric acid A 10 per cent solution is prepared at frequent intervals* preferably each day Since the acid deteriorates rapidly at room temperature it should be dissolved in cold water and stored in a refrigerator

4 Enzymes A 10 per cent solution of Takadiastase or Clarase is prepared at frequent intervals preferably each day The preparation should yield a pale yellow solution and it should dissolve readily with very little residue The insoluble material contains considerable quantities of thiamine The solution is centrifuged and the clear supernatant solution is kept in a refrigerator

Collection and Preparation of Samples

1 *Blood* Blood is collected and is prevented from clotting with a minimum of heparin or oxalate The sample is cooled immediately and the analysis is made within a few hours of collection Five ml are transferred to an NPN tube the volume is adjusted to about 5 ml and 20 ml of N HCl are added.

Feces Samples are collected in large glass vessels and stored in the cold. For digestion 5 gm samples are weighed on filter papers and filter paper and samples are transferred to NPN tubes Three ml of N HCl are added and the volume is adjusted to 35 ml (Because of many factors which affect digestion absorption and intestinal motility the composition of the stool may vary considerably from day to day even when the human subject is receiving a constant weighed diet In order to be of any significance the collection should be carried out for at least 4 days The storage bottles must be kept cold Bacterial synthesis of thiamine due to growth of bacteria may greatly increase the vitamin content of the sample if it is not immediately analyzed or if it is not kept cold)

3 Cereals and dry samples of food. The sample is finely ground by suitable means

4 Fresh tissues animal and plant. The sample is rapidly cut into small strips or chunks A weighed portion is homogenized with approximately 100 ml of water in the *Waring Blendor It is then transferred quantitatively to a volumetric flask* and the volume is brought to the mark A suitable aliquot is transferred to an NPN tube

Procedure —Extraction

1 The finely ground or homogenized sample is weighed or measured by means of a syringe pipette It is transferred to an NPN tube the volume is brought to the 35 ml mark and a measured volume of N HCl is added. The quantity of acid to be added should be determined for each type of material Enough acid should be

added so that the acidity after 10 or more minutes in the boiling water bath is approximately pH 3 The contents of the tube are thoroughly emulsified by means of a J shaped stirring rod They are then heated 30 minutes with frequent stirring in the boiling water bath

2 The tube is now cooled in a cold bath of running water after which 1 drop of caprylic alcohol and a volume of M $NaHCO$ which is equivalent to the N HCl is added The contents after stirring should have a reaction of pH 4 to 5

3 Several blank tubes which contain equal volumes of N HCl and M $NaHCO$ are prepared at this time They should be carried through the subsequent operations along with the sample

Procedure—Hydrolysis of Bound Thiamine

1 *Add 2 ml of enzyme solution* More of enzyme preparation is added to samples which contain much starch or viscous material The tube is incubated 2 hours in a water bath at 40 to 50 C The mixture is stirred frequently (Some samples *like blood* required *only 1 hour* of incubation Long incubation may introduce serious errors due to bacterial growth The temperature should be kept near 45 C in order to prevent such growth)

2 *The tube is cooled in a bath of running water*

Procedure—Precipitation of Protein

1 The contents of the tube are transferred quantitatively to a volumetric flask of °50 ml capacity 20 ml of HPO are added and the volume is brought to the mark Sufficient time should be given to allow diffusion of the vitamin from the particles into the solution

2 Filter or centrifuge (The analysis may be discontinued at this time The flasks may stand overnight in the refrigerator)

3 Blood and samples which contain less than 1 microgram of thiamine are treated somewhat differently Ten ml (or le s if the protein content is small) of HPO solution are added the volume is brought to 50 ml and the contents are mixed thoroughly The supernatant solution is poured off after centrifugation the re idue is emulsifed with *2 5 ml HPO solution (using the J rod) and the volume* is again brought to the 50 ml mark The clear supernatant solution is added to the first supernatant

Procedure—Adsorption Elution, Oxidation, Extraction

1 The necessary adsorption columns are prepared as under thiamine in urine

2 An aliquot of the extract sufficient to contain 1 to 5 mcg of thiamine is adjusted to pH 3 5 by means of 0 1 N NaOH with bromcresol green as indicator The extract is then passed through the column After passage of all of the extract the flask is rinsed with several succe sive portions of about 5 ml each of distilled water and the washings are sent through the column The fluid is allowed to drain completely after each of the washings

3 With the tip of the column in an NPN tube four 5 ml portions of °5°' KCl are sent through the column *Finally enough ‿o% KCl is sent through to bring the* volume to exactly 25 ml

4 Five ml portions of eluate (10 for blood) are added to extraction vessels and the *rest of the procedure is* followed as under thiamine in urine

Calculation

1 Prepare calibration curves with standard thiamine as described under urine

2 mcg total thiamine/100 gm initial substance =

$$\frac{(\text{mcg in cuvette} - \text{blank}) \times (\text{total eluate}) \times (\text{total extract}) \times 100}{(\text{ml eluate used}) \times (\text{ml extract used}) \times (\text{gm sample})}$$

Example

A sample of feces weighed 4 701 gm The fluorescence of the unknown was equivalent to 0 60 mcg thiamine in the tube that of the blank was equivalent to 0 07 mcg *The sample had been diluted to 50 ml of which 50 ml had been sent through* the zeolite The zeolite had been eluted with 25 ml of KCl of which 5 ml samples were analyzed.

mcg total thiamine/100 gm feces =

$$\frac{(0\,60 - 0\,07) \times (5) \times (250) \times 100}{(5) \times (50) \times (4\,701)} = 8 \text{ mcg/100 gm}$$

Precautions

Same as for thiamine in urine

4 N^1 Methylnicotinamide in Urine

Reference

Coulson R A Ellinger P and Holden M A Method for the Estimation of N cotinamide Methochloride in Urine Biochem J 38 150 154 (1944)

Principle

N^1 methylnicotinamide a metabolic product of niacin breaks down in alkaline solu*tion to a fluorescent compound which is soluble in isobutyl alcohol*

Apparatus

Same as for thiamine in urine

Reagents

Same as for thiamine in urine with a few additions

1 Standard N^1 methylnicotinamide Dissolve 10 mg of the pure substance in 50 ml of 1% acetic acid (40 mcg/ml of solution)

Procedure

Same as for thiamine in urine down to the oxidation step After eluting the column with KCl proceed as follows

1 To 5 ml of KCl eluate add 10 ml of isobutyl alcohol
   Add 0 7 ml of 15% KOH stopper and shake immediately for at least 50 times
   Stand as least 10 minutes before reading
3 Read as in thiamine analysis with quinine standard at 60 (Maximum fluorescence develops slowly so that a period of standing is required before reading)

Standard Curve

Run a series of standard N^1 methylnicotinamide solutions The range should be 40 gamma to 00 gamma per sample with intervals of 40 gamma

Calculation

$$\text{mg } N^1 \text{ methylnicotinamide/100 ml urine} = \frac{(\text{mcg in cuvette}) \times (\text{total eluate}) \times 100}{(1000) \times (\text{ml aliquot}) \times (\text{ml urine})}$$

Example

A reading of 30 on the galvanometer was equivalent to 43 mcg in the cuvette The *blank was equivalent to 10 mcg Five ml of urine were used for analysis and* eluted with 5 ml of KCl all of which was used for analysis.

$$\text{mg } N^1 \text{ methylnicotinamide/100 ml urine} = \frac{(43 - 10) \times 5 \times 100}{1000 \times 5 \times 5} = 0\,66 \text{ mg/100 ml}$$

Precautions

This is a method fraught with inaccuracies At every step there are significant losses Hence the results must be considered only semi quantitative

5 Riboflavin in Urine, Blood, Food and Feces

A. IN URINE

Reference

Connor, R T and Straub G J Combined Determination of Riboflavin and Thiamin in Food Products Ind and Eng Chem Anal Ed 13 385 389 (June) 1941

Principle

Riboflavin is measured fluorometrically after interfering substances are destroyed An internal standard is used and the blank is determined after reduction of riboflavin to the leuko form which is not fluorescent

Apparatus

1 Photofluorometer Coleman Model 12 or 12B
2 Cuvettes Coleman 19 × 150 mm
3 Syringe pipettes one and 2 ml
4 A number of 25 ml graduated cylinders with ground glass stoppers

Reagents

1 Buffer solution pH 4 7 Dissolve 111 gm of sodium acetate plus 54 4 ml of glacial acetic acid in 1000 ml of water
2 Potassium permanganate a 4% solution in water
3 Hydrogen peroxide a 3% solution in water
4 Standard riboflavin solutions Prepare a stock solution (100 mcg/ml) by dissolving 10 mg of standard in exactly 100 ml of 3% acetic acid Store in a dark bottle in a refrigerator This solution is stable for six months For a working standard (1 mcg/ml) dilute the stock 1 to 100 with water

TABLE 10

RIBOFLAVIN IN URINE

(For analyses in which 1 ml of urine is analyzed in 10 ml of solution A is galvanometer reading with unknown B with 1 mcg riboflavin internal standard and C with blank This table gives approximately correct values in the range of normal urine)

A C (ACROSS)	18	0	[illegible]	24	26	8	30	32	34	36
B A (DOWN)	mcg B /ml URINE									
20	82	89	98	1 07	1 15	1 3	1 9	1 38	1 45	1 54
1	78	85	94	1 0	1 10	1 18	1 24	1 32	1 39	1 46
22	75	82	90	98	1 05	1 13	1 19	1 27	1 33	1 41
23	1	79	87	94	1 01	1 09	1 13	1 22	1 28	1 35
4	69	75	84	90	98	1 04	1 10	1 17	1 24	1 31
5	66	72	81	88	94	1 00	1 07	1 13	1 20	1 6
26	64	70	8	84	90	97	1 03	1 09	1 15	1 22
27	6	68	75	81	88	94	1 00	1 06	1 12	1 18
8	60	65	73	79	85	90	97	1 02	1 08	1 14
29	59	63	70	76	82	88	93	99	1 05	1 10
30	56	61	68	74	79	85	90	96	1 0	1 07
31	54	60	66	72	7[illegible]	82	88	93	99	1 04
32	52	58	65	70	75	79	85	91	96	1 01
33	51	56	63	68	73		82	88	93	99
34	50	55	61	66	71	75	80	85	91	96
35	48	53	59	64	69	73	78	82	88	93
36	47	52	57	62	67	71	75	80	86	91
37	46	51	56	61	65	69	73	78	84	88
38	45	49	54	59	64	67	71	75	8	86
39	44	48	53	58	62	65	69	74	80	84
40	43	47	52	56	61	64	67	7	77	8
41	42	46	51	55	59	63	66	70	75	80

5 Sodium fluorescein reference standard Dilute 10 mg of sodium fluorescein to 1000 ml in 3% acetic acid For a working standard (0 5 mcg/ml) dilute the stock 1 to 20 with water
6 Pure sodium hydrosulfite powdered

Procedure

1 Pipette exactly 1 ml urine plus 3 ml buffer solution into a 25 ml graduated stoppered cylinder
2 Add one or two drops of 4% KMnO until a pink or violet color persists for one minute
3 Then add two to 4 drops of H O (3%) until the color disappears
4 Make up to 10 ml with buffer solution mix and transfer to cuvettes
5 For a reagent blank use 1 ml distilled water instead of 1 ml of urine
6 Read samples within one hour (keeping in dark meanwhile) as follows
   a Warm up instrument for 15 minutes before using Use B filter
   b Balance at zero and set galvanometer at 25 with a sample of the dilute fluorescein reference in the cuvette
   c Read the unknown (Reading A)
   d To the sample add exactly 1 ml of standard B solution (1 0 mcg B)
   e Mix thoroughly with stirring rod and read immediately (Reading B)
   f To the same cuvette now add a small amount of sodium hydrosulfite on the end of a spatula stirring until dissolved
   g Read immediately (Reading C)

Calculation

1 Reading A represents the galvanometer deflection due to riboflavin plus blank in 10 ml

Reading B represents the galvanometer deflection due to added riboflavin in 11 ml

Reading C represents the galvanometer reading due to the blank alone in 11 ml

TABLE 10 (CONT D)

A C (ACROSS)	38	40	4	44	46	48	50	5	54	56
B A (DOWN)	mcg B_2/ml URINE									
20	1 59	1 6	1 74	1 80	1 88	1 94	00	07	14	0
-1	1 53	1 60	1 67	1 3	1 80	1 88	1 9	00	0	2 1
	1 47	1 54	1 60	1 67	1 74	1 81	1 85	1 9	1 98	04
3	1 41	1 48	1 54	1 61	1 67	1 74	1 78	1 85	1 91	1 9
4	1 36	1 43	1 49	1 55	1 61	1 68	1	1 78	1 84	1 90
5	1 31	1 38	1 43	1 50	1 56	1 6	1 66	1 73	1 78	1 84
6	1	1 33	1 39	1 4	1 51	1 57	1 61	1 67	1 73	1 78
7	1 23	1 9	1 35	1 40	1 46	1 5	1 56	1 6	1 67	1 3
8	1 19	1 5	1 30	1 36	1 41	1 4	1 51	1 5	1 62	1 68
9	1 15	1 1	1	1 32	1 3	1 43	1 47	1 5	1 58	1 63
30	1 1	1 18	1	1 8	1 33	1 3	1 43	1 48	1 53	1 58
31	1 09	1 14	1 19	1 4	1 30	1 3	1 39	1 44	1 49	1 54
3	1 06	1 11	1 16	1 21	1 26	1 31	1 35	1 40	1 45	1 50
33	1 03	1 08	1 13	1 18	1 23	1 8	1 31	1 36	1 41	1 46
34	1 00	1 05	1 10	1 15	1 19	1 5	1 8	1 33	1 37	1 42
35	8	1 03	1 0	1 1	1 16	1	1 5	1 30	1 34	1 38
36	95	1 00	1 04	1 09	1 13	1 19	1	1 27	1 31	1 35
37	93	98	1 01	1 06	1 11	1 16	1 19	1 4	1 8	1 3
38	91	95	99	1 03	1 08	1 13	1 16	1 21	1 5	1 9
39	88	93	9	1 01	1 06	1 10	1 13	1 18	1	1 6
40	86	91	95	99	1 03	1 08	1 10	1 15	1 19	1 23
41	84	88	93	97	1 01	1 06	1 08	1 13	1 17	1 20

2 mcg riboflavin/100 ml urine =

$$\frac{(A - C \times \frac{11}{10})}{(B \times \frac{11}{10} - A)} \times \text{mcg riboflavin added} \times \frac{\text{ml dilution}}{\text{ml urine}} \times 100$$

Example

1 One ml of urine was diluted to 10 and read 15 0 (Reading A) After 1 mcg of riboflavin was added the reading was 50 0 (Reading B) After hydrosulfite the reading was 5 0 (Reading C)

mcg riboflavin/100 ml urine =

$$\frac{15\ 0 - 5 \times \frac{11}{10}}{50\ 0 \times \frac{11}{10} - 15\ 0} \times 1 \times \frac{10}{1} \times 100 = 275 \text{ mcg/100 ml}$$

2 A table (Table 10) has been constructed which *for most practical purposes gives* a good approximation to the correct value over most of the normal range The assumptions made are (a) the blank is usually small so that blank × $\frac{11}{10}$ is equivalent to blank (b) Reading B − Reading A is almost constant so that B × $\frac{11}{10}$ − A is *also almost constant*

Precautions

1 Keep samples in dark as much as possible
2 Because of the danger of reoxidation read immediately after adding hydrosulfite

B IN BLOOD FOOD AND FECES

Reference

Same as for riboflavin in urine

Principle

The determination consists of

(1) liberation of bound alloxazine nucleotides by means of heat and acid
(2) *clarification of the sample and conversion of alloxazine nucleotides into* riboflavin by means of Clarase or Takadiastase
(3) removal of many impurities by adsorption on Supersorb and subsequent elution with pyridine solution at pH 5
(4) oxidation of impurities in the eluate by means of KMnO and
(5) determination of fluorescence

Apparatus

Same as for thiamine in blood food and feces

Reagents

1 *Hydrochloric* acid approx N Conc HCl (sp gr 1 19) is diluted 1_ times, or 83 ml are diluted to a volume of 1000 ml in distilled water
2 Sodium bicarbonate approx N *Dilute 84 gm NaHCO to 1000 ml*
3 Isoamyl alcohol
4 Enzyme solution A 10% solution of Takadiastase (Parke Davis *Detroit) or* Clarase (Takamine Laboratories, Clifton N J) is prepared at frequent intervals preferably each day The preparation should yield a pale yellow solution and it

should dissolve readily with very little residue The solution is centrifuged and the clear supernatant solution is kept in the refrigerator when not in use

5 Supersorb (Floridin Co, Warren Pa) The adsorbent as bought contains much fine material which must be removed It also must be activated before it can be used One pound or more is covered with a 1 per cent solution of acetic acid (in a large round bottom flask with short neck or 3 liter Erlenmeyer flask) The mixture is stirred very briefly by rotation allowed to stand until most of the adsorbent has settled and the supernatant solution is then poured off This is repeated twice It is similarly washed by decantation several times with distilled water after which it is transferred to a large Buchner funnel and washed further with distilled water The Supersorb is then dried at room temperature in a large pan In order to have the material in its maximum expanded state before use a small quantity is kept moistened in a wide mouthed bottle

6 Pyridine solution Approximately 500 ml of distilled water 200 ml of redistilled pyridine and 0 ml of glacial acetic acid are transferred to a 1000 ml volumetric flask after which the volume is made to the mark with distilled water

7 Potassium permanganate Dissolve 31 6 gm $KMnO_4$ in one l distilled water Keep this approximately N solution in a brown dropping bottle

8 Hydrogen peroxide USP 3% Keep in a brown dropping bottle

9 Riboflavin stock solution The reference standard (Dr E F Cook 43rd St and Woodland Ave Philadelphia) is dried for 4 hours over phosphorus pentoxide 40 mg are transferred to a flask of 1000 ml capacity Water to about 800 ml and 100 ml of approximately N H_2SO_4 are added The solution is frequently shaken and is kept in the refrigerator or in a cool dark place until all of the vitamin is dissolved The contents are warmed to a temperature of 0 C after which the volume is brought to the mark The flask is protected from light in double brown paper bags If kept in the refrigerator the stock standard solution is stable for at least 6 months (40 mcg/ml)

10 Riboflavin working solution Dilute 2 ml of the stock solution to a volume of 50 ml or 10 ml to a volume of 250 ml Five ml of this intermediate standard and 25 ml (graduated cylinder) of pyridine solution are transferred to a 50 ml volumetric flask and the volume is brought to the mark The sensitivity is adjusted to a galvanometer reading of 50 (0 16 mcg/ml)

11 Sodium hydrosulfite solution (Lycopon) Since sodium hydrosulfite reacts rapidly with atmospheric oxygen solutions must be prepared immediately before use they must not be unnecessarily shaken the surfaces should be small and solutions should be used not longer than 2 hours To 5 ml of ice cold water contained in an NPN tube are added 1 gm of sodium hydrosulfite and 1 gm of $NaHCO_3$

1 Capryl c alcohol

Collection and Preparation of Samples

The conditions for collection and preparation of samples are the same as described in the directions for the determination of thiamine by the thiochrome method.

Special precautions must be taken to avoid unnecessary exposure of samples to strong light especially to sunlight

Procedure

1 The finely ground or homogenized sample is weighed or measured by means of a pipette It is transferred to an NPN tube the volume is brought to the 35 ml mark and a measured volume of N HCl (from 3 to 5 ml) is added Enough acid should be added to bring the acidity of the boiled sample to pH 1 to 2 The quantity of acid to be added should be determined for each type of material

2 The contents of the tube are thoroughly emulsified by means of a J shaped stirring rod They are then heated 30 minutes to 1 hour with frequent stirring especially during the first 10 minutes in the boiling water bath
3 The tube is now cooled in a cold bath of running water, after which 1 drop of caprylic alcohol and a volume of M $NaHCO_3$ which is equivalent to the N HCl are added. The contents after stirring should have a reaction of pH 4 to 5
4 Several blank tubes which contain equal volumes of N HCl and M $NaHCO_3$ are prepared at this time They should be carried through the subsequent operations along with the sample
5 Add 2 0 ml of Clarase or Takadiastase solution More of the enzyme solution is added to samples which contain much starch or viscous material The tube is incubated 2 hours in a water bath at 40 to 50 C the contents being stirred frequently
6 The tube is cooled in a bath of running water after which the contents are transferred quantitatively to a volumetric flask of 250 ml capacity The volume is brought to the mark At this point the analysis may be discontinued and the flask may be stored in the refrigerator
7 The solution is allowed to settle overnight or if analyzed immediately it is centrifuged Filtration may cause large and variable errors due to adsorption of the vitamin by the paper If filtered the first 25 ml of the solution should be discarded
8 The columns are filled with Supersorb as described in directions for the determination of thiamine
9 A suitable aliquot of the clear supernatant solution is transferred to a 150 ml extraction flask Bromcresol green indicator and enough water to bring the volume to about 100 ml are added The reaction is then adjusted to approximately pH 4 5 by means of 0 1 N HCl or NaOH
10 The extract is passed through the column After passage of all the extract the flask is rinsed with two successive portions of about 5 ml each of distilled water and the washings are sent through the column The fluid is allowed to drain completely after each of the washings The container below the column is removed and the liquid which has passed through the column is discarded A calibrated NPN tube is put in its place below the column
11 Small portions of pyridine solution are now add d until exactly 25 ml of eluate have been collected at which point the elution is discontinued
12 Oxidation of impurities in the pyridine eluate Permanganate solution is added drop by drop until an excess is present as indicated by a violet color After an interval of 1 to 5 minutes H_2O_2 is added drop by drop until the solution is completely clean Usually 4 drops of $KMnO_4$ and 8 drops of H_2O_2 solutions are sufficient
13 The volume is brought to the 50 ml mark and the solution is mixed by means of a J stirring rod
14 Several tubes or cuvettes containing the standard should be prepared and they should be used alternately to prevent rise of temperature It should be emphasized that the fluorescence changes with temperature The standards should therefore be kept under the same conditions as the solutions which are to be examined The standard tubes should be stoppered if they are to be used over a period of time
15 The cuvette or tube is filled to the mark The sensitivity of the apparatus is set by means of the standard after which the reading is taken with the unknown solution This is reading A An excess (0 2 ml) of sodium hydrosulfite solution is then added the contents are quickly mixed and the reading is made immediately This is reading B (The solution should be mixed with gentle rotation after addition of the hydrosulfite Violent mixing produces bubbles which scatter the light and thus give high results Riboflavin becomes nonfluorescent in

stantly B ologi al materials conta n many substances whose fluorescence is slowly destroyed by the hydrosulfite The reading therefore should be made immediately after addition and mixing of the reagent)

Standardization

1 Known quant t es of the standard are adsorbed on Supersorb eluted with pyridine and treated with KMnO as described above Water blanks should be ncluded in the ser es The readings corrected by the blank are then plotted as mcg riboflav n/ml solut on in the cuvette

The presence of light adsorbing substances in the eluate may markedly alter the results even though the recovery of added riboflavin may be quantitative Milk urine and feces for example conta n such sub tances However their effect is almost entirely eliminated by the adsorption and the KMnO oxidation. The recovery should be d term ned for every type of sample If repeated analyses yield quantitative recovery of added riboflavin it is not necessary to make such determinations in subsequent analyses It is suggested however that the re covery be tested at frequent intervals as a check on the accuracy of the tech nique

Calculation

1 mcg riboflavin/ml solut on in cuvette =

$$\frac{(\text{unknown reading A} - \text{B}) - (\text{Blank read ng A} - \text{B})}{\%\ \text{recovery}} \times 100 \times \text{calibration factor}$$

2 mcg r boflavin /100 ml original material =

$$\frac{(\text{mcg riboflav n/ml in cuvette}) \times (\text{ml eluate}) \times (\text{total ml extract}) \times 100}{(\text{ml extract through column}) \times (\text{gm sample})}$$

Example

We ght of food 4 gm Dilution after hydrolysis 50 ml Total volume adsorbed 50 ml Total olume elution 5 ml Reading A 55 Reading B 11 Blank read ing A 9 Blank eading B 5 Recovery 94%

1 m g riboflavin/ml solution in cuvette =

$$\frac{(55\ 11) - (9\ 5)}{94} \times 100 \times \frac{0\ 16}{50} = \frac{40}{94} \times 0\ 3 = 0\ 136\ \text{mcg/ml}$$

$$\text{mcg r boflavin/100 gm food} = \frac{0\ 136 \times 5 \times 50 \times 100}{50 \times 4} = 404\ \text{mcg/100 gm}$$

Precautions

Same as for r boflavin in ur ne

6 Ascorbic Acid in Whole Blood Serum and Urine

A. WHOLE BLOOD AND SERUM (MACROMETHOD)

Reference

Roe J H and Kuether C. A Th Dete m at on of As orbic Acid in Whole Blood and Urine Thro gh the 4 dinitrophenylhydrazine Derivat ve of Dehydro ascorbic Ac d J Biol Chem 147 399 40 (Feb) 1943

Principle

A d washed No it converts a co b c ac l to dehydroa corbic acid. In the pres ence 4 dinitrophenylh d az ne dehydroascorbic acid is coupled with the phenyl hydra ne In the p e e o of strong sulfuric acid a colored con pound is formed from the hydra o e

Apparatus

1. Coleman Jr Spectrophotometer Model 6
2. Cuvettes 19 × 150 mm
3. Centrifuge tubes capacity of 50 ml
4. Incubator set at 37 C
5. A quantity of 125 ml Erlenmeyer flasks
6. An electric centrifuge
7. A quantity of 50 ml centrifuge tubes
8. Quantitative filter paper Whatman 40
9. An ice bath

Reagents

1. 2 4 dinitrophenylhydrazine reagent Dissolve 2 gm of hydrazine in 100 ml of 9 N sulfuric acid (3 parts of water and one part of concentrated sulfuric acid) Filter
2. Acid washed Norit Place 200 gm Norit in a large flask Add 1 liter of 10% HCl and heat to boiling Filter with suction Remove Norit cake to a large beaker and add 1 liter distilled water Stir thoroughly and filter Repeat until washings give a negative or very faint test for ferric iron. (Test filtrate with 1% potassium ferrocyanide)
   Dry Norit cake in oven overnight at 110 120 C (Norit is an activated charcoal)
3. Trichloracetic acid a 6% solution in water
4. Sulfuric acid 85% solution To 100 ml of distilled water add 900 ml of concentrated sulfuric acid (sp gr 1 84)
5. Thiourea a 10% solution Dissolve 10 gm thiourea in 100 ml of 50% (by volume) aqueous ethyl alcohol This reagent keeps satisfactorily for two months but to check it see that it readily reduces HgCl or KMnO
6. Standard Vitamin C Dissolve exactly 100 mg in 100 ml of 5% acetic acid (1 mg/ml) Dilute 10 to 100 with 6% trichloracetic acid (100 mcg/ml) Add 1 ml of thiourea before making to volume

Procedure

1. Pipette 15 ml of 6% trichloracetic acid into a 50 ml Pyrex centrifuge tube Add 5 ml of whole blood (plasma) drop by drop and with a glass rod stir until fine suspension is produced
2. Let stand for at least 5 minutes and centrifuge
3. Transfer supernatant to Pyrex test tube Add 0 75 gm (3 scoops) acid washed Norit and shake vigorously Filter through 9 cm filter paper
4. Place 4 ml Norit filtrate in each of two matched photoelectric colorimeter tubes Add 1 drop of 10% thiourea to each
5. Hold one tube as reserve for blank
6. Add 1 ml hydrazine reagent to other
7. Place second tube in constant temperature bath at 37 C Keep tube immersed for exactly 3 hours
8. Remove tube and place this and the blank in an ice bath
9. To each tube add slowly 5 ml of 85% sulfuric acid Add the acid drop by drop from the burette taking at least one to one and a half minutes for total addition Mix thoroughly with tubes immersed
10. Add 1 ml hydrazine reagent to blank.
11. Remove and after 30 minutes at room temperature wipe the tubes clean and dry
12. Transfer to cuvettes
13. Set blank at 100% T at 540 mμ and read unknowns

Standard Curve

Prepare a standard curve by running a series of ascorbic acid standards through the method The range should be from 0 to 35 mcg per tube at intervals of 5 mcg Plot on semilog paper

Calculation

$$\text{mg total ascorbic acid/100 ml serum} = \frac{\text{mcg in cuvette} \times \text{total dilution} \times 100}{1000 \times \text{ml aliquot} \times \text{ml serum}}$$

Example

A reading of 77 5% T was equivalent to 10 mcg of ascorbic acid in the cuvette Five ml of blood were diluted to 0 ml of which 4 ml were taken for analysis

$$\text{mg total ascorbic acid/100 ml serum} = \frac{10 \times 20 \times 100}{1000 \times 4 \times 5} = 1\ 0\ \text{mg/100 ml}$$

Precautions

1 Norit must be free from ferric iron whose ions may irreversibly oxidize as corbic acid Ferric ions al o react with hydrazine reagent producing interfer ng colors
Thiourea by producing a reducing environment protects against interference by ferric ons and other ox dizing substances The coupling reaction is not appreciably slowed by th ourea.
3 The final color is stable for 40 minutes After 18 hours the maximum fad ng is ° 5 points on the galvanometer scale
4 Glucose xylos fructose and glucuronic acid do not interfere in ordinary phys ologic and pathologic ranges

B IN URINE (MACROMETHOD)

Reference Principle Apparatus and Reagents

Same as for ascorbic acid in blood

Procedure

1 To a 1 5 ml Erlenmeyer flask add exactly 5 ml of urine
° Add 5 ml of 6% trichlo acetic acid (This stabil zes the ascorbic acid during extraction.)
3 Add 0.5 gm acid washed Norit and shake vigorously (Norit oxidizes ascorbic ac d to dehydro form.)
4 Filter
5 Pipette 4 ml of filtrate into two colorimeter tubes
6 Add 1 drop of thiourea to both
7 Add 1 ml of 4 dinitrophenylhydrazine to one excluding the blank
8 Mix and incubate for 3 hours at 37 C
9 Remove and place in ice bath.
10 Add 5 ml of 85% sulfuric acid slowly so as not to ra se the temperature (1 to 1½ minutes)
11 Add 1 ml of hydrazine reagent to the blank
1 Set blank at 100% T and read all specimens at 540 mμ

Standard Curve

Same as for ascorbic acid in blood.

Calculation

$$\text{mg ascorbic acid/100 ml urine} = \frac{\text{mcg in cuvette} \times \text{total ml d lut on} \times 100}{1000 \times \text{ml aliquot} \times \text{ml urine}}$$

Example

A reading of 7 5% T was equ valent to 10 mcg ascorbic acid in the cuvette Five ml of urine had been diluted to 10 ml of wh ch 4 ml aliquots were analyzed

$$\text{mg total ascorbic acid/100 ml urine} = \frac{10 \times 10 \times 100}{1000 \times 4 \times } = 0\ 0\ \text{mg /100 ml}$$

Precautions

Same as for ascorbic ac d in blood

WHOLE BLOOD AND SERUM (MICROMETHOD)

Reference

Bessey O A Vitamin Methods Vol 1, Academic Press Inc Publishers Edited by P Gyorgy New York, pages 303 305 1950

Lowry O H, Lopez J A and Bessey O A The Determination of Ascorbic Acid in Small Amounts of Blood Serum, J Biol Chem 160 609 615 (Oct) 1945

Principle

Same as for the method of Roe and Kuether

Apparatus

1 A Coleman Jr Spectrophotometer or a Beckman Spectrophotometer Model DU
2 A quantity of cuvettes 12 × 75 mm
3 A cuvette holder for 12 × 75 mm cuvettes
4 A water bath or an incubator set at 38 C
5 An ice bath
6 Quantitative filter paper or an electric centrifuge
7 A quantity of 15 ml round bottom centrifuge tubes
8 A quantity of #1 solid rubber stoppers

Reagents

1 A 2 % solution of 2 4 dinitrophenylhydrazine made up in 10 N sulfuric acid.
2 Thiourea a 5% solution in water This solution keeps well for one month if left at 4 C
3 Copper sulfate ($CuSO_4$ $5H_2O$) an 0 6% solution made up in 0 01 N sulfuric acid This solution is stable indefinitely
4 Sulfuric acid 65% solution Dilute 70 ml of concentrated sulfuric acid with 30 ml of distilled water Keep in the refrigerator
5 Trichloracetic acid 4% solution
6 Trichloracetic acid a 5% aqueous solution
7 For the combined reagent dilute 18 ml of the 2 4 dinitrophenylhydrazine solution 1 ml of copper sulfate solution and 1 ml of thiourea (This solution is stable for at least one week and should be centrifuged unless crystal clear)

Procedure

1 Pipette 4 ml of 5% trichloracetic acid into a 15 ml round bottom centrifuge tube
2 Add exactly 1 ml of whole blood or serum stopper and mix
3 Centrifuge at 3000 r p m for 10 minutes
4 Pipette exactly 1 ml of the aliquot into each of two small test tubes
5 Into one tube add exactly 0 25 ml of the mixed thiourea copper sulfate and 2 4 dinitrophenylhydrazine reagent
6 Mix by tapping and incubate for 4 hours at 38 C
7 Chill in ice water and add 1 5 ml of cold 65% sulfuric acid solution The cold acid may be added at once
8 Now add 0 25 ml of the mixed reagent to the blank tube and mix by shaking
9 Set the blank at 100% T at 520 mμ and read the unknowns
10 Treat 1 ml of standard ascorbic acid solution (in 4% trichloracetic acid solution) in the same way These solutions should cover a range of 3 to 21 mcg/ml at intervals of 3 mcg

Calculation

$$\text{mg total ascorbic acid/100 ml of serum} = \frac{\text{mcg in cuvette} \times \text{total dilution} \times 100}{1000 \times \text{ml aliquot} \times \text{ml serum}}$$

Example

One ml of blood was diluted to 5 ml of which one ml was used in the analyses Ascorbic acid in the cuvette was 3 0 mcg

Therefore

$$\text{mg total ascorbic acid/100 ml of blood} = \frac{3 \times 5 \times 100}{1000 \times 1 \times 1} = 1.5$$

Precautions

1 The final concentration of acid is critical in this method and must be watched carefully

Variations in the concentration of thiourea will cause large variations in the final color

3 The age of the trichloracetic acid appears to be immaterial.

7 Ascorbic Acid in Tissue

Reference

Roe J H Mills M B, Oesterling M J and Damron C M The Determination of Diketo l Gulonic Acid Dehydro l Ascorbic Acid and l Ascorb c acid in the Same Tissue Extract by the 2 4 Dinitrophenylhydraz ne Method J Biol. Chem 174 201 08 (May) 1948

Principle

Same as for serum ascorbic acid

Apparatus

The same as for serum ascorbic acid with the addition of

1 A mortar and pestle

An H S generator

3 Carbon dioxide gas

Reagents

The same as for serum ascorbic acid with the addition of

1 Metaphosphoric acid 5% in water

Stannous chloride solution Dis olve 10 gm stannous chloride in 100 ml 5% metaphosphoric acid

3 Thiourea powdered

4 Bromine

Preparation of Tissue

1 Weigh accurately a sample of tissue of from to 5 gm This sample is ground in a mortar with ml of 10% $SnCl_2$ solution Add 100 ml of 5% metaphosphoric acid and mix thoroughly (Do not u e a Waring Blendor or homogenizing apparatus that would introduce increased amounts of oxygen into the slurry) The slurry is thoroughly mixed and filtered. (A sol tion w th a d lut on of 1 part of tissue in 100 parts of extract is desirable and in dilutions greater than this a concentration 1 to 10 per ml of total AA DHA and DKA is recommended It is necessary to work rapidly to prevent changes in partition of the three compounds. A conversion of DHA to DKA of 7 percent in a solution contain ng 0 5 percent SnCl – 5 percent HPO was observed in 1 hour at room temperature

Procedure for Diketo l-Gulonic Acid (DGA)

1 Place 100 ml of the original $SnCl_2$ HPO filtrate in a large tube. Introduce a gas filter tube with a s nte ed glass filter about 0 mm in d ameter and pass H_2S through for 15 minutes

2 To 40 ml of the H_2S saturated extract add 0 4 gm of powdered thiourea shake until dissolved and filter
3 Bubble CO into the filtrate for 5 minutes
4 Pipette 4 ml aliquots into each of three colorimeter tubes
5 To two tubes add 1 ml of 2 4 dinitrophenylhydrazine The third tube will be a blank
6 Place in water bath at 37 C for 6 hours
7 From here on the analysis is the same as for serum ascorbic acid.

Procedure for the Sum of Dehydro-l Ascorbic Acid (DHA) and Diketo-l-Gulonic Acid (DGA)

1 Pipette 4 ml aliquots of the original SnCl HPO filtrate into each of three cuvettes
2 Add 1 ml of 2, 4 dinitrophenylhydrazine to two of the tubes the third being used as a blank
3 Place in a water bath at 37 C for 6 hours
4 Continue as for serum ascorbic acid.

Procedure for the Sum of l Ascorbic Acid (AA) Dehydro l Ascorbic Acid (DHA) and Diketo-l-Gulonic Acid (DGA)

1 The remainder of the H_2S saturated solution is filtered to remove SnS (thiourea is not added) into a test tube suitable for bubbling gas Air is drawn through a water trap before passing through the H S solution to minimize volume changes Sufficient bromine is added to color the solution Excess bromine is removed by bubbling air through the solution
2 Add powdered thiourea in sufficient quantities to make a 1% solution
3 Pipette 4 ml aliquots into three colorimeter tubes
4 Add 1 ml of 2 4 dinitrophenylhydrazine to two tubes the third being used as a blank.
5 Place in a water bath at 37 C for 6 hours
6 Continue as for serum ascorbic acid

Preparation of Calibration Curves

1 Because the rate of coupling is influenced by the acid solution used and the anti toxidant present it is important in preparing a calibration curve to use the same conc ntration of HPO and thiourea or SnCl as is used in the procedure followed for the unknown A calibration curve is made with standard dehydro ascorbic acid solution prepared by bromine oxidation of ascorbic acid as directed by Roe and Oe terling The standards are made up in 5 percent HPO solution contain ing 1 percent of thiourea The tubes are incubated for 6 hours at 3, C The curve obta ned with 1 percent thiourea is practically the same as that obtained with 0 5 percent $SnCl_2$ Therefore calculations of DHA DGA and AA are all made with the same standard curve

Calculation

1 All three sub tan es are of practically the same molecular weight and are ex pressed in te ms of mg ascorbic acid, according to the procedure outlined under ascorbic acid.
2 DGA is measured directly
3 *DHA = (DHA + DGA)* − DGA
4. AA = (AA + DHA + DGA) − (DHA + DGA)

Precautions

1 Speed in handling ti ue and timing of reactions are the most important aspects of this method.

8 Rapid Field Methods for Ascorbic Acid, Riboflavin, Thiamine and N[1] Methylnicotinamide

Reference

1 For ascorbic acid see Farmer C J and Abt A F Proc Soc Exper Biol & Med 34 146 150 1936

2 For riboflavin thiam ne and N[1] methylnicot namide see Johnson R E Sargent F II Robinson P F and Con olazio C F Estimation of Riboflavin Thi amine and N[1] methylnicotinamide Rapid Field Methods Ind and Eng Chem. Anal. Ed 17 384 387 (June) 1945

A. ASCORBIC ACID IN SERUM AND URINE.

Principle

The blue dye 6 d chlorophenolindophenol in acid solution is decolorized by as corbic acid. An aqueous solution of the dye standardized against known amounts of ascorbic acid can therefore be used to titrate samples of serum and urine

Apparatus

1 Burette 5 ml capacity graduated to 05 ml
Round bottom te t tubes 15 ml capacity
3 Pipettes to del ver exactly 0 1 ml 0 5 ml and approx 3 5 ml Automatic syringe pipettes are the most satisfactory type
4 Su table titration bench with good daylight or dayl ght electric shining against a white background.

Reagents

1 Metaphosphoric acid approximately 3% and 6% in aqueous solution This takes a long time to d ssolve and one should start making it several hours before use It keeps for several day
Buffered solution of 6 dichlorophenolindophenol In the field this should be p epared from a dry mixture weighed out in the laboratory of 15 mg of the dye 1 8 gm KH_2PO_4 and 8 gm of Na_2HPO_4 all mixed together and stored dry in small envelopes One envelope is d ssolved in about 500 ml of water either d s tilled or tap if the latter s blank is known to be low The aqueous solution of buffered dye will keep for se eral days but changes its standardization so that it must be standardized daily as described below
3 Ascorb c acid 100 mg water soluble tablets prepared by reputable drug houses are satisfactory for making a standard solut on but each fresh lot of tablets must be assayed before use In a 100 ml mix ng cylinder place about 0 ml of 3% metaphosphoric acid add 1 tablet ascorbic acid and fill accurately to the mark with water The tablet is dissolved by inversion After complete solu tion 1 ml of this strong standard is p petted accurately into a 100 ml mixing cylinder containing about 0 ml of metaphosphoric acid. The cylinder is filled accurately to the mark and mixing is effected by invers on This weak stand ard contains 1 0 mg of ascorb c acid per 100 ml and is used for standardizing the dye as described below

Procedure

1 Before each day s work the dye must be standard zed against a fresh solution of ascorbic acid 4 ml of the weak standard ascorbic acid are pipetted accurately into a 15 ml test tube Dye is added from the burette until the first pink color lasting 30 sec is reached Satisfactory dupl cate estimations must be obtained before any fu ther steps are taken The titration figure should be about 3 ml
A reagent blank must be run before each day s work 4 ml of the 3% meta pho phor c acid solution are pipetted accurately into a test tube and are titrated

against the dye For satisfactory results the titration figure must be below 0.2 ml. If it is higher there is something wrong with the reagents most probably the water and this error must be corrected before any further steps are taken.

3 Estimation of vitamin C in urine Urine is collected and stored with oxalic acid as a stabilizer as described in the section on urine. In the case of fasting specimens exactly 0 5 ml is pipetted into a test tube and approximately 3.5 ml of 3% metaphosphoric acid is added. In the case of loaded specimens exactly 0 1 ml of urine is used. In either case dye is added from the burette rather rapidly The first definite pink lasting 15 sec is taken as the end point and no notice is taken of fading after this stage is reached. There are in urine reducing substances other than ascorbic acid which reduce the dye somewhat more slowly than does ascorbic acid. Rapid titration is therefore absolutely essential

4 Estimation in serum. Exactly 2 ml of serum are added to exactly 6 ml of 6% metaphosphoric acid in a 15 ml centrifuge tube The tube is stoppered with a clean rubber stopper and is gently inverted several times Do not shake vigorously because of froth formation. The tube is centrifuged at high speed and exactly 4 ml of the supernatant fluid are pipetted into a test tube Titration is carried out directly on the supernatant fluid, which already contains metaphosphoric acid.

Calculations

A. Standardization of the dye

$$\text{mg ascorbic acid equivalent to 1 ml of dye} = \frac{0\,04}{\text{titration in ml} - \text{reagent blank in ml}}$$

B Calculation for serum.
mg ascorbic acid in 100 ml = (titration figure in ml − reagent blank in ml) × equivalent of dye × 100

C Calculation for fasting urine
mg ascorbic acid in 100 ml = (titration figure in ml − reagent blank in ml) × equivalent of dye × 200

D Calculation for loaded urine
mg ascorbic acid in 100 ml = (titration figure in ml − reagent blank in ml) × equivalent of dye × 1000

Examples

1 Reagent blank = 0 10 3 °5 ml dye titrated 4 ml of weak standard therefore 1 ml dye = 0 04 ÷ (3 °4 − 0 10) = 0 0127 mg ascorbic acid.

2 Titration figure for serum = 0 85 ml. Therefore serum contains (0 85 − 0 10) × 0 01°, × 100 = 1 0 mg ascorbic acid per 100 ml

3 Titration figure for fasting urine = 0 55 ml dye Therefore fasting urine contains (0 5o − 0 10) × 0 0127 × 200 = 1 1 mg ascorbic acid in 100 ml.

4 Titration figure for loaded urine = 1 00 ml dye Therefore loaded urine contains (1 00 − 0 10) × 0 01.7 × 1000 = 11 4 mg ascorbic acid in 100 ml.

5 Use of constant factors During a single day the standardization of the dye does not change Hence calculation is facilitated by computing the Factor F representing the constant number by which the titration figure is multiplied to give the result directly in terms of mg ascorbic acid per 100 ml of serum fasting urine or loaded urine In example 1 above 1 ml of dye = 0 01°7 mg ascorbic acid.

Hence F for serum = (0 012) × (100) = 1.27
F for fasting urine = (0 01.7) × (°00) = 2 54
F for loaded urine = (0 01.7) × (1000) = 12 7

ASCORBIC ACID

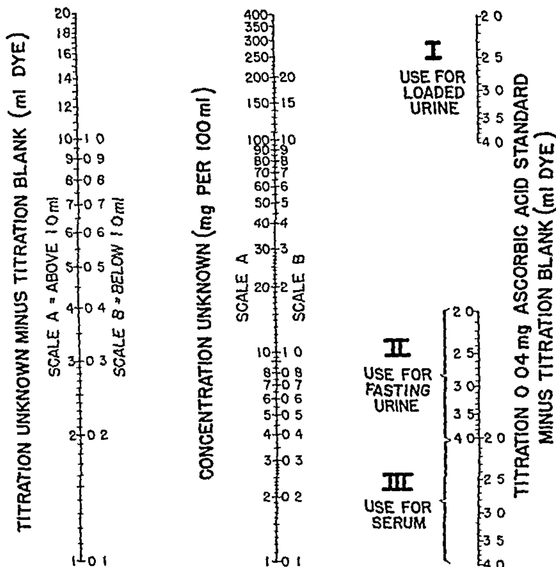


Fig 2 —Line chart for ascorbic acid. For use, see text.

6 Use of line chart The nomogram (Fig 22) is for serum fasting urine and loaded urine. A string is stretched between the left hand line (titration minus blank) and the right hand line (titration of 0 04 mg ascorbic acid minus blank) and the concentration of ascorbic acid in 100 ml of serum fasting urine or loaded urine is read off on the middle line Section I of the right hand line is for use with loaded urine Section II for fasting urine and Section III of serum when the procedure is exactly as described above

Precautions

1 The reagents must be standardized at least once daily and in hot weather twice a day

2. Titration of urine must be carried on as rapidly as is consistent with accurate work. Serum need not be titrated so rapidly because it does not contain significant amounts of nonascorbic acid reducing substances

3 It is emphasized that even in individuals whose body stores of vitamin C are very low the apparent urine level by this method never reaches 0 because of the presence in urine of reducing substances which are not ascorbic acid.

B RIBOFLAVIN IN URINE

Principle

Riboflavin in urine is extracted into isobutyl alcohol after a preliminary treatment in the presence of pyridine and acetic acid with potassium permanganate followed by hydrogen peroxide The isobutyl layer is cleared with anhydrous sodium sulfate and is read fluorometrically

Apparatus

1 Apparatus for collecting and storing urine as described in Section II.

2 *Apparatus for visual fluorometry as described in the section on fluorometry* The comparator rack for reading solutions of riboflavin should have fixed on the side nearest the eye a suitable yellow glass filter with maximal transmission at 560 *microns.*

3 At least two dozen glass stoppered test tubes of 10 ml capacity These are conveniently made from standard 10 ml glass stoppered footed mixing cylinders by removing the foot with a sharp tap of a tack hammer and rounding the bottom in the blast lamp

Reagents (Assuming 100 specimens)

1 Reagents for storing urine as described in *Section II.*

2 A mixture of 1 part pyridine and 1 part glacial acetic acid. This should be made up fresh each day 50 ml for 100 specimens

3 *5 ml aqueous 4% potassium permanganate*

4. 10 ml aqueous 3% hydrogen peroxide This keeps well and the potency of any batch should be such that two drops destroy 1 drop of 4% permanganate in the presence of acetic acid.

5 Isobutyl alcohol of low blank. Normal butyl alcohol is equally satisfactory Each fresh batch of alcohol should be examined before the ultraviolet light and must be redistilled if there is perceptible fluorescence

6 Anhydrous sodium sulfate 40 gm.

7 Water soluble tablets of riboflavin 5 mg for standards Each batch must be assayed before use

Procedure

1. All manipulations should be carried out in diffuse light. Only direct sunlight destroys riboflavin rapidly enough to make extreme precautions necessary against light.

In the case of fast ng urine exactly 0 5 ml of urine is pipetted into a 10 ml glass stoppered test tube In the case of loaded urine exactly 0 1 ml is added and 0 4 ml of water to keep volume relations constant

3 0 5 ml of pyridine acetic acid mixture is added and mixed by gentle shaking of the rack

4 1 drop of potassium permanganate is added and mixed by a few gentle shakes of the rack At this point the tubes may stand anywhere from ½ to 5 minutes without affecting results

5 Two drops of hydrogen peroxide are added and mixed by gentle shaking of the rack The permanganate should be destroyed within a few seconds If it is not another drop of hydrogen peroxide may be necessary In temperatures near freezing gentle warming of the tube may be necessary This is conveniently done in a water bath at about 70 F

6 Exactly 1 5 ml of isobutyl alcohol are added by means of either an ordinary pipette or preferably from an automatic syringe pipette which avoids getting unpleasant vapors in the mouth The glass stopper is inserted and the tube is shaken vigorously by hand w th an up and down motion 25 times The distribution coefficient of riboflavin between the aqueous phase and the alcohol pyridine acetic acid phase is such that more shaking has been proved to be unnecessary

7 The test tube is allowed to stand in the rack until the aqueous phase is nearly separated. This usually takes from 1 to 5 minutes being faster the h gher the temperature

8 A small wide mouthed funnel is placed in the neck of the tube after the glass stopper has been removed and a small spatula of sodium sulfate is added to the tube A gentle rotary motion imparted to the tube assists clearing of the alcohol layer

9 The tube is allowed to stand in the rack until it is water clear which usually takes one to two minutes However a few turns on a hand centrifuge will materially hasten this step

10 Approximately 1 ml of the alcohol layer is transferred to a standard thin walled Pyrex test tube of d mensions 10 × 75 mm This s best achieved w th an auto mat c syringe p pette as described under fluorometry but any pipette can be used

11 Reading is conducted before the ultraviolet lamp as described und r fluorometry The alcohol solut ons are stable in the dark for at least hours even at h gh temperatures and therefore there is no hurry in reading them Make sure that standards and unknowns are at approximately the same temperature

1 After this initial reading if one wishes to obtain true riboflavin the tubes must be exposed to strong ultraviolet light for an hour or more Where sunshine can be counted on it may be u ed but usually an ultraviolet lamp is es ential If this step is omitted sati factory comparative results are still obtained s nce the amount of fluorescence not destroyed by ultraviolet light is remarkably constant at about 50% of the init al value Following load ng tests because of the high concentrations of riboflavin exposure to ultraviolet light may be safely om tted and a constant factor used

Standard Solutions

Two types of sta dards will be described The first and theoretically best is made by running aqueous solutions through the whole method Such standards tend to to le un table and when a large numb r of est mations have to be done the sec ond type of sta dard is recommended. The e consist of aqueous solutions of ribo fl v n which are co siderably more stable but somewhat less desirable otherwise Both types of stand rd must be checked at least once a day for deterioration

A Standards run through the whole method

1 Prepare two liters of water containing 0 3 ml of conc H SO per liter Acidulated water is used in all subsequent steps because *riboflavin is most stable in 0 01 N* H SO

2 *Dissolve one 5 mg tablet* in *exactly 1 liter* of acidulated water This strong standard contains 5 mcg riboflavin per ml

3 In a glass stoppered mixing cylinder make standard solutions by adding from a pipette the amount of strong standard shown in the *table below* diluting to exactly 25 ml with acidulated water mixing transferring to a dry *1 oz* amber bottle *for storage* rinsing the mixing cylinder with water and starting the next dilution in the same cylinder

CONCENTRATION RIBOFLAVIN REQUIRED mcg/100 ml	STRONG STANDARD REQUIRED ml	
0	0	In each case dilute to 25 ml with acidulated water
25	1 25	
50	2 5	
75	3 75	
100	5 0	
125	6 25	
150	7 50	
200	10 0	
250	12 50	
300	15 0	

4 Run ½ ml samples of the standards through the method as described in steps 1 to 12 above The final alcohol layers should be placed in clean dry glass stoppered *standard test tubes the racks of which should be clearly marked with the value* of the initial standard solutions

B Aqueous standards for use with large numbers of estimations

1 Prepare two liters of water containing 0 3 ml of conc H SO

2 Dissolve one 5 mg pill in exactly one liter of acidulated water This strong standard contains 5 mcg riboflavin per ml

3 Prepare a working standard by diluting exactly 30 ml of strong standard ? to exactly 50 ml with acidulated water

4 Prepare a series of aqueous standards as in step A 3 but using the following table which is constructed on the assumptions that the over all recovery *of riboflavin in* the method is 80% and that 0 5 ml samples of urine are to be used

STANDARD DESIRED mcg RIBOFLAVIN PER 100 ml	WORKING STANDARD REQUIRED ml	
0	0	In each case dilute to exactly 25 ml with acidulated water
25	2 0	
50	4 0	
75	6 5	
100	8 5	
125	10 5	
150	12 5	
200	16 5	
250	21 0	
300	25 0	

5 Store these in brown botttles in a cool place They will keep for one week

6 For use as standards the aqueous solutions as they stand *should be placed directly* in the standard tubes without running them through the method The racks of the tubes should be labeled with the numbers in the left hand column *The values then represent* mcg of riboflavin per 100 ml of urine when 0 5 ml samples are analyzed

7 Replace the standards in the tubes morning and afternoon when large numbers of *estimations are being done*

Calculations

The calculations for fasting urines are as follows

mcg/100 ml urine = (1st reading – reading after 1 hr in UV light) If the sample has not been exposed to sunlight or ultraviolet light mcg/100 ml urine = (1st reading) × (0 5)

The calculations for loaded urines are as follows

mcg/100 ml urine = (1st reading – reading after 1 hr in UV light) × 5 If the sample has not been exposed to sunlight or ultraviolet light mcg/100 ml urine = (1st reading × 5) – (1 5)

Examples

Fasting Urine First reading 50 Reading after sunshine 1 5 Therefore mcg riboflavin/100 ml = (2ɔ0 – 1 5) = 1 5

Loaded Urine First reading 00 Reading after sunshine 5 Therefore mcg riboflavin/100 ml — (00 – ?5) × 5 = 875

Precautions

1 The commonest source of error in this method is the instability of the standards These must be checked at least every day and when many readings are being made twice a day

Our experience has been that an ultraviolet bulb is far preferable to sunlight because of the extreme variability of the sunshine in climates where there is intermittent cloudiness

3 Standards and unknowns must be at the same temperature when read

THIAMINE AND N¹ METHYLNICOTINAMIDE

Principle

These two substances are estimated by methods so similar that they are best described together except for the last steps Both compounds are absorbed from urine on activated zeolite and eluted with potassium chloride Thiamine is converted to thiochrome by ferricyanide in alkaline solution N¹ methylnicotinamide (hereafter referred to as NMN) is itself nonfluorescent but is converted to the fluorescent Factor F by mere addition of alkali. The thiochrome and Factor F are shaken into isobutyl alcohol and are estimated fluorometrically

Apparatus

1 Apparatus for collection and storage of urine as described in Section II

Apparatus for visual fluorometry as described in Section III F

3 Glass stoppered 10 ml test tubes as under riboflavin

4 Beakers pipettes funnels and if available automatic syringe pipettes as under riboflavin

Reagents

1 Activated zeolite This requires considerable time patience and sometimes experimentation in order to get a satisfactory product which will settle out rapidly and also absorb thiamine well. Commercial zeolite is crushed in a stone crusher or suitable mortar (In our experience permutit and decalso are as good as zeolite) That portion of the product which will pass a 100 mesh sieve is saved and suspended in 1% acetic acid in distilled water in large cylinders. The granules which settle to the bottom rapidly that is in about 3 minutes are separated from the lighter particles by decanting These heavier particles are boiled three times with 1% acetic acid with settling and decanting between fresh additions of acetic acid The product is washed with distilled water and dried in the oven at 110 C. It should be a white homogeneous powder and when about ?00 mg are shaken with water in a 10 ml mixing cylinder all the particles should settle rapidly to the bottom in not more than 2 min We have encountered great vari

bility from batch to batch When test runs with synthetic thiamine are made recovery is sometimes poor and the product may have to be reactivated by the technique of Najjar and Wood (1940) Sometimes addition of ferricyanide results in the formation of Prussian blue In such cases the product has to be washed with warm fairly strong solutions of hydrochloric or nitric acid When a satisfactory product is finally obtained its activity remains constant for months if it is kept dry

2 Aqueous potassium chloride approximately 25% *At temperatures near freezing* the solubility properties necessitate lower concentrations

3 Aqueous sodium hydroxide approximately 15%

4 Bromcresol green, 1% in water

5 Aqueous potassium ferricyanide 0 25% This should be made up fresh every day, *most conveniently from weighed samples stored dry in small stoppered test tubes*

6 Isobutyl alcohol This must have a blank of essentially zero and each new batch has to be tested before use It can be recovered by redistillation of the residue after analysis Normal butyl alcohol can be used instead of isobutyl

7 Acidulated water This is most conveniently made up a gallon at a time Approximately 20 ml of glacial acid are added to a gallon of distilled water Tap water of low blank may be used but is usually inferior to distilled water

Procedure

1 Urine is collected and stored as described in Section II. Thiamine and NMN are stable for at least a week even at high temperatures provided the pH of the sample is between 3 and 6 This pH is almost always attained with the oxalic acid in the storage bottle

2 *The acidity of the urine is checked soon after collection* with bromcresol green as external indicator The acidity is correct when the indicator turns any shade of green or yellow If it is blue add glacial acetic acid drop by drop to the storage bottle until the acidity is correct

3 Steps 4 through 8 which follow are identical for NMN and thiamine It is therefore convenient to run both at the same time but with separate aliquots until the final stages which will be differentiated below 2 ml of fasting urine are pipetted into a glass stoppered test tube or 0 5 ml of loaded urine

4 About 200 mg of zeolite are delivered from a small spatula into the tube most *easily through a small funnel and mixed by about ten rapid vigorous* shakes of the rack The time of standing at this stage is not critical up to ½ hr No more than ten shakes are required

5 Approximately 8 ml of acidulated water are added to the tube from a large syringe and the tube is mixed by inversion 10 times with the finger as a stopper After a few seconds standing in the rack until *the particles of zeolite have begun* to settle the few particles adherent to the top and sides are washed down by closing the top with a clean finger and administering a single sharp upward jerk The tube is replaced in the rack until the zeolite particles have settled to the bottom In urine containing oxalic acid there is usually slight permanent turbidity which is disregarded in deciding when the zeolite has settled

6 The supernatant fluid is withdrawn by means of suction through a long needle of stainless steel and is discarded A mechanical vacuum pump is highly desirable but a 50 or 100 ml syringe may be used if necessary

7 *Approximately 8 ml of* acidulated water are added and the processes of standing inversion and removal of the supernatant fluid are repeated This washing step is critical in both the estimation of NMN and thiamine but for different reasons In the case of thiamine if washing is not effective the fluorescence obtained in the final step is off color with a silvery blue admixture to the true thiochrome mauve and reading is *wholly unsatisfactory In our experience tap water* can be

used instead of distilled water but it may be necessary in this case to add more acetic acid to the water or even sometimes to wash three times before the thiochrome color is satisfactory. Thiamine is firmly bound to the zeolite and repeated washings remove the interfering substances without affecting the thiamine. Experimentation determines how many washings with the available water supply are required in order to get satisfactory results for thiamine. In the case of NMN in contrast to thiamine each washing diminishes the final fluorescence. Apparently NMN is relatively easily washed off the zeolite. In making routine surveys it is therefore essential to adopt and follow scrupulously a fixed technique of washing in order to be able to compare values for NMN from one place to another.

8 0.5 ml of potassium chloride is added to the tube and the rack is shaken gently so as to mix the zeolite with the potassium chloride but not to streak the zeolite far up the sides. The time necessary for elution is short, not exceeding 30 seconds. From this point on the estimations of NMN and thiamine are different.

Final steps for thiamine

9 0.1 ml of potassium ferricyanide is added and mixed by a few gentle shakes of the rack.

10 0.5 ml of sodium hydroxide is added and mixed with a gentle shake of the rack. The time necessary for completing the oxidation from thiamine to thiochrome is only a few seconds. Once formed in this mixture it is stable for at least ½ hour.

11 Add [illegible] ml of isobutyl alcohol. The glass stopper is inserted and the tube is shaken vigorously [illegible] times up and down its long axis. This number of shakes insures complete distribution of thiochrome between the alcohol and the aqueous phases. More shaking is superfluous.

12 The tube is allowed to stand in the rack until separation of the phases is almost complete and is then centrifuged if necessary a few seconds in a hand centrifuge to insure complete clearing.

13 About 1 ml of the supernatant fluid is transferred to a small test tube by means of a pipette or a syringe and needle.

14 The fluorescence is matched against standards in the visual comparator as described in the section on fluorometry, care being taken that standards and unknowns are at approximately the same temperature.

Final steps for N¹ methylnicotinamide

9 [illegible] ml of isobutyl alcohol are delivered into the tube. No mixing is necessary at this point.

10 0.5 ml of sodium hydroxide is added, the tube is stoppered with its glass stopper and is shaken [illegible] times as under thiamine. The addition of sodium hydroxide should be followed by shaking at once because Factor F in aqueous alkaline solution breaks down to nonfluorescent end products within a very few minutes but is stable in the isobutyl layer for days.

11 The tube is replaced in the rack and when separation of the phases is more or less complete it is centrifuged if necessary for a few seconds in a hand centrifuge.

12 1 ml of the supernatant layer is transferred to small test tube as under thiamine.

13 Fluorescence is matched in the comparator against standard solutions as described in the section under fluorometry. The color of F_2 develops relatively slowly after step 10. It is absolutely necessary to allow at least 5 minutes between step 10 and the final reading. The development of color goes on at the same rate either at step 11 or at step 12, becomes maximal in 5 minutes and then remains constant for at least an hour. Care should be taken that standards and unknowns are at approximately the same temperature.

Standards

A For thiamine

In order to compensate for variability in the reagents standards are made by running solutions of thiamine hydrochloride through the method

1 Prepare 2 liters of acidulated distilled water by adding approximately 20 ml of glacial acetic to 2 liters of distilled water This acidulated water is used in all subsequent steps since the vitamin is maximally stable at about pH 4 and is quickly destroyed above pH 7

2 One 5 mg tablet of thiamine hydrochloride is dissolved in exactly one liter of acidulated water This strong standard contains 5 mcg of thiamine hydrochloride per ml

3 Dilute exactly 75 ml of strong standard to exactly 250 ml with acidulated water This working standard contains 1 5 mcg of thiamine hydrochloride per ml

4 Using a mixing cylinder and graduated pipettes make up the following series of standards

CONCENTRATION OF THIAMINE HYDROCHLORIDE DESIRED (mcg/100 ml)	WORKING STANDARD REQUIRED (ml)	
0	0	In each case dilute to exactly 25 ml with acidulated water
5	0 83	
10	1 67	
15	2 5	
20	3 33	
30	5 0	
50	8 3	
75	12 5	
100	16 7	
150	25 0	

5 The above standards are stored in 1 oz amber bottles in a dark cool place. They keep for at least 2 weeks

6 Two ml of each of the series are run through the thiamine method One ml of the isobutyl layer is placed in the standard comparator tube the rack of which is clearly labeled with the concentration of the original solution Standards must be checked daily for deterioration

B For N^1 methylnicotinamide

1 Dissolve 10 0 mg N^1 methylnicotinamide chloride in exactly 133 ml of 1% acetic acid in water

2 Make up the following for storage in 1 oz brown bottles

CONCENTRATION DESIRED (mg PER 100 ml)	ml N^1 METHYLNICOTINAMIDE	
0	0	In each case dilute to exactly 25 0 ml with 1% acetic acid.
0 25	0 8	
0 50	1 7	
0 75	2 5	
1 0	3 3	
1 5	5 0	
2 5	8 3	
3 75	12 5	
5 0	16 6	
7 5	25 0	

3 Store these standards in a cool place They keep for 1 2 weeks N^1 methyl nicotinamide is not fluorescent but is converted to F in alkaline isobutyl alcohol

Calculations

1 Fasting specimens of urine
mcg of thiamine per 100 ml and mg of NMN per 100 ml = reading on rack of standard.

Loaded urine
meg of thiamine per 100 ml and mg of NMN per 100 ml = reading on rack of standard x 4.

3 On theoretical grounds most of the standard laboratory methods subtract F from thiochrome in calculating thiamine We do not make this correction in the present method because of the demon tration by Najjar and Ketron (1944) that F is about 80% destroyed by the addition of ferricyanide Therefore only when the F is *large* in comparison with B is correct on for F neces ary This has very rarely happened in our field surveys

Examples

1 Fasting urine
Readings B_{4} F 1 5 Therefore mcg B per 100 ml urine = 30 and mg NMN per 100 ml = 1 5

2 Loaded urine
Readings B 30 F 1 5 Therefore mcg *B* p r 100 ml urine = 4 × 40 = 160 mg NMN per 100 ml urine = 4 × 4 0 = 16 0

Precautions

A For both thiam ne and NMN

1 The chief sources of error in these methods are two the difficulty of getting a sat sfactory preparat on of zeolite and the washing stages Both of these diffi culties have to be settled by experimentation

2 Once prepared the standards for thiamine usually are stable for one to two weeks They should be checked every day or two against freshly prepared standards

3 The standards and unknown should be at the same temperature when read be cause fluorescence is strongly affected by changes in tem perature

B For thiamine

1 For urine of subjects taking quinine or atabrine pre liminary treatment of the specimen is essential
(a) Pour approximately 7 ml of urine into a centrifuge tube
(b) Add approximately 3 5 ml of isobutyl alcohol and about 10 mg Norite (¼ of a small spatula) The exact amount of charcoal is critical as too much will destroy the thiam ne
(c) Close with thumb and shake 5 times.
(d) Centrifuge and u e ml samples of the water layer *below* the obutyl layer

C For N methylnicot namide

1 In the final step be sure to u e the following order 0 5 ml KCl mix 2 ml iso-butyl alcohol 0 ½ ml NaOH shake at once. *F is* un table in watery alkaline solutions but stable in the isobutyl layer
After the shaking with NaOH F develops relat vely slowly in the isobutyl layer *At least* 5 minutes mu t elapse after the shaking before the specimen is read You may either let the glass stoppered tubes s t for 5 minutes or else you may draw off the isobutyl layer into the read ng tubes and read after 5 minutes. A m nimum of 5 minutes waiting is absolutely essential. The color is stable up to one hour so that speed in reading is not *necessary*

2 For urine of subjects taking quinine or atabrine prelim nary treatment with charcoal is e sent al The procedure is
(a) Pour approx 5 ml of urine into a centrifuge tube.
(b) Add approx 20 mg Norite A (about ½ of a small spatula) The exact amount is not critical for F but enough is e sential
(c) Close with thumb and shake 25 times.
(d) Centrifuge and use 2 ml samples of supernatant fluid.

9 Vitamins and Nutrition Miscellaneous References

Books

American Medical Association The Vitamins A Symposium Chicago 1939 American Medical Association

Association of Official Agricultural Chemists Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists ed 6 Washington 1945 AOAC

Association of Vitamin Chemists Inc Methods of Vitamin Assay, New York 1947 Interscience

Bicknell F and Prescott F The Vitamins in Medicine ed 2 reprinted 1947 New York 1947 Grune and Stratton

Coward K H The Biological Standardization of the Vitamins ed 2 London 1947 Bailliere Tindall and Cox

Dann W J and Satterfield G H Biological Symposia XII Estimation of the Vitamins New York 1947 *Jacques Cattell Press vol XII*

György P Vitamin Methods New York 1950 Academic Press Inc Publishers vol. I

McLester J S Nutrition and Diet in Health and Disease ed 5 Philadelphia and London 1949, W B Saunders Company

Rosenberg H R Chemistry and Physiology of the Vitamins New York 1942 Interscience

Sahyun M Proteins and Amino Acids in Nutrition New York 1948 Reinhold Publishers Vol xvi

Sherman H C Chemistry of Food and Nutrition ed 7 New York 1946 The Macmillan Company

Williams R R and Spies T D Vitamin B (Thiamin) and Its Use in Medicine New York 1940 The MacMillan Company

U S *Pharmacopoeal Conventions The Pharmacopoeia of the U S of America 13th* Revision (USP XIII) Washington 1947

Youmans J B Nutritional Deficiencies Diagnosis and Treatment ed 2 Philadelphia Montreal London 1943 J B Lippincott Company

Ascorbic Acid

Adams J Acker M and Frediani H. A Iodimetric Titrimetric Determination of Ascorbic Acid J Am Pharmaceut Assoc Sci Ed 36 (6) 170 172 (July) 1947

Bessey O A *A Method for the Determination of Small Quantities of Ascorbic Acid and* Dehydroascorbic Acid in Turbid and Colored Solutions in the Presence of Other Reducing Substances J Biol Chem 126 (2) 771 784 (Dec) 1938

Farmer C J and Abt A F Determination of Reduced Ascorbic Acid in Small Amounts of *Blood Proc Soc Exp Biol & Med 34 146 150 (March) 1936*

Hochberg M Melnick D and Oser B L Photometric Determination of Reduced and Total Ascorbic Acid Ind Eng Chem (Anal Ed) 15 (3) 182 188 (March) 1943

Huelin F E and Stephens I M The Influence of Ferrous Iron in the Determination of Ascorbic Acid *Australian J Exper Biol & M Sc 25 (1) 17 23 (March) 1947*

King C G Chemical Methods for Determination of Vitamin C Ind Eng Chem (Anal Ed) 13 (4) 225 227 (April) 1941

Mills M B and Roe J H A Critical Study of Proposed Modifications of the Roe and Kuether Method *for the Determination of Ascorbic Acid With Further Contributions* to the Chemistry of This Procedure J Biol Chem 170 (1) 159 164 (Sept) 1947

Mindlin R L and Butler A M The Determination of Ascorbic Acid in Plasma A Macromethod and Micromethod J Biol Chem 122 673 686 (Feb) 1938

Pecover V The Microdetermination of Ascorbic Acid in Blood Plasma Australian J Exper Biol & M Sc 25 (2) 175 178 (June) 1947

Carotene

Johnson R M and Baumann C A Studies on the Reaction of Certain Carotenoids With Antimony Trichloride *J* Biol Chem 169 (1) 83 90 (June) 1947

Choline

Marenzi A D and Cardini C E The Colorimetric Determination of Choline J Biol Chem 147 (2) 363 370 (Feb) 1943

Entenman C Taurog A and Chaikoff I L The Determination of Choline in Phospholipids J Biol Chem 155 (1) 13 18 (Sept) 1944

Folic Acid

Allfrey V Teply L J Geffen C and King C G A Fluorometric Method for the Determination of Pteroylglutamic Acid J Biol Chem 178 465 481 (March) 1949

Glazko A J and Wolf L M Colorimetric Determination of Folic Acid and Adenine Arch. Biochem. 21 241 242 (March) 1949

Nicotinic Acid

Banerjee S Ghosh N C and Bhattacharya G A Chemical Method of the Estimation of Nicotinic Acid in the Presence of Sugar J Biol Chem 172 (2) 495 499 (Feb) 1948

Harris L J and Raymond W D Assessment of the Level of Nutrition A Method for the Estimation of Nicotinic Acid in Urine Biochem J 33 (1) 037 051 (Dec) 1939

Mueller A and Fox S H Chemical Determination of Niacin J Biol Chem 167 (1) 291 9 (Jan) 1947

Perlzweig W A Levy E D and Sarett H P Nicotinic Acid Derivatives in Human Urine and Their Determination J Biol Chem 136 7 9 745 (Dec) 1940

Teeri A F and Shimer S R A Colorimetric Determination of Nicotinic Acid J Biol Chem 153 (1) 307 311 (April) 1944

Pyridone of N1 Methylnicotinamide

Rosen F Perlzweig W A and Handler P A Fluorometric Assay for N1 Methyl 6 pyridone 3 carboxylamide Proc Fed Soc 7 181 (March) 1948

Rosen F Perlzweig W A and Leder I G A Fluorometric Method for the Determination of the 6 Pyridone of N1 Methylnicotinamide in Urine J Biol Chem. 179 157 168 (May) 1949

4 Pyridoxic Acid

Huff, J W and Perlzweig W A A Product of Oxidative Metabolism of Pyridoxine 2 Methyl 3 hydroxy 4 carboxy 5 hydroxymethylpyridine (4 pyridoxic acid) I Isolation From Urine Structure and Synthesis J Biol Chem 155 345 355 (Sept) 1944

Pyridoxine

Hochberg H, Melnick D and Oser B L Chemical Determination of Pyridoxine in Biological Materials and Pharmaceutical Products The Multiple Nature of Vitamin B J Biol Chem 155 (1) 119 1 8 (Sept) 1944

Riboflavin

Emmett A D Bird O D Brown R A Peacock G and Vandenbelt J M Determination of Vitamin B_2 (Riboflavin) Comparison of Bioassay Microbiological and Fluorometric Methods Ind. Eng Chem (Anal. Ed.) 13 (4) 19 2 1 (April) 1941

Loy H W Jr Report on Riboflavin (Microbiological)—Revised Method (Chemical)—Fluorometric Method Asso of Offic Agric Chemists 32 (3) 461 464 1949

Morell D B and Slater E C The Fluorimetric Determination of Riboflavin in Urine Biochem. J 40 (5/6) 6 657 1946

Rubin S H De Ritter E Schuman R L and Bauernfeind J C Determination of Riboflavin in Low Potency Foods and Feeds Ind. Eng Chem (Anal Ed) 17 (3) 1 6 140 (March) 1945

Slater E C and Morell D B A Modification of the Fluorimetric Method of Determining Riboflavin in Biological Materials Biochem J 40 644 65 1946

Thiamine

Egaña E and Meiklejohn A P The Estimation of Thiamine in Urine J Biol Chem 141 859 870 (Dec) 1941

Friedemann T E and Kmieciak T C The Determination of Thiamine in Blood J Lab & Clin Med. 28 1 6 1 68 (July) 1943

Hennessy, D J Chemical Methods for the Determination of Vitamin B Ind. Eng Chem (Anal Ed) 13 16 18 (April) 1941

Hennessy D J, and Cerecedo L. R. The Determination of Free and Phosphorylated Thiamin by a Modified Thiochrome Assay J Am Chem. Soc. 61 (1) 1 9 183 (Jan) 1939

Hochberg M Melnick D, and Oser B L Simplified Colorimetric Determination of Thiamine in Cereal Product Cereal Chem 22 ( ) 83 90 (March) 1945

Mickelsen O Condiff, H and Keys, A. Determination of Thiamine in Urine by Means of the Thiochrome Technique J Biol. Chem 160 (1) 361 370 (Sept) 1945

Tocopherol

Beneze B The Determination of Tocopherol by the Color Reagent of Furter and Meyer Z Physiol Chem. 282 15 157 194

Minot A S The Determination of Tocopherol in Blood Serum J Lab & Clin. Med. 29 (7) 780 (July) 1944.

Quaife M L and Biehler R A Simplified Hydrogenation Technique for the Determination of Blood Plasma Tocopherols J Biol. Chem 159 (3) 663-665 (Aug) 1945

Quaife M L, and Harris P L The Chemical Estimation of Tocopherols in Blood Plasma J Biol Chem 156 (2) 499 505 (Dec) 1944
Quaife M L Scrimshaw, N S and Lowry O H A Micromethod for Assay of Total Tocopherols in Blood Serum J Biol Chem 180 1–29 1235 (Oct) 1949

Trigonelline

Kodicek E and Wang Y L A New Method for the Estimation of Trigonelline in Urine and Foodstuffs Nature 148 –3 24 (July) 1941

Vitamin A and Carotene

Adlersberg D Kann S Maurer A P Newerly K Winternitz W and Sobotka, H Studies on Serum Carotene in Man Am J Digest Dis 16 (9) 333 337 (Sept) 1949
Clausen S W, and McCoord A B The Carotinoids and Vitamin A in the Blood J Pediat 13 (–) 635 650 (Nov) 1938
Kawasaki, C and Suenaga S Colorimetric Estimation of Vitamin A by Glycero-dichlorohydrin Reagent J Pharm Soc Japan 69 460 463 1949
Koch W and Kaplan D A Simultaneous Carr Price Reaction for the Determination of Vitamin A J Biol Chem 173 (1) 363 –69 (March) 1948
Thompson, S Y A Photoelectric Spectrophotometer Suitable for the Measurement of Vitamin A by the Antimony Trichloride Reaction, British J Nut 3 (1) 43 50 1949

Vitamin D

DeWitt J B and Sullivan M X Spectrophotometric Procedure for Quantitative Estimation of Vitamin D Ind Eng Chem (Anal Ed) 18 117 119 (Feb) 1946
Muller P B *The Colorimetric Determination of Vitamins A D and β Carotene With* Particular Regard to Vitamin D Helv Chim Acta 30 (5) 117– 1190 1949
Sobel A E Mayer A M and Kramer B A New Colorimetric Reaction of Vitamin D and D and Their Provitamins Ind Eng Chem (Anal Ed) 17 (3) 160 165 (March) 1945

SECTION IV

BIOCHEMICAL PROCEDURES (Continued)

F HORMONES

1 Ketosteroids in Urine

Reference

Consolazio W. V. and Talbott J. H. The Extraction and Determination of 17-Ketosteroids in Urine J Endocrinol. 27 355-359 (Sept.) 1940

Principle

The steroid linked to glucuronic acid is set free by hydrolyzing with hydrochloric acid and is extracted with carbon tetrachloride. Interfering substances are washed out and the resulting extract is equilibrated with alkaline meta dinitrobenzene. The final color is measured.

Apparatus

1. One continuous extractor unit. (See reference Made by MacAlaster Bicknell Company Cambridge Mass. See Fig 3)
2. Steam hydrolyzer (According to Consolazio and Talbott.)
3. All glass still to fit round bottom extraction flask
4. Automatic syringe pipette 0.[illegible] ml capacity
5. Six pear shaped separatory funnels ([illegible]0 ml capacity) with interchangeable ground glass stoppers.
6. Swivel separatory funnel rack
7. Coleman Jr Spectrophotometer
8. A quantity of 19 x 1[illegible]0 mm cuvettes.
9. A quantity of unglazed porcelain
10. A water bath set at [illegible]5 C.
11. An electric centrifuge
12. A steam bath

Reagents

1. Potassium hydroxide (KOH) exactly [illegible] N
2. Ethyl alcohol (EtOH) aldehyde free
3. Meta dinitrobenzene $C_6H_4(NO_2)_2$ 2% Dissolve [illegible]0 gm in [illegible]0 ml of EtOH. Heat to 40 C and add 100 ml of 2 N NaOH After 5 minutes cool and add [illegible]00 ml of water Filter and wash thoroughly with water Dry by suction. Recrystallize twice from 1[illegible]0 ml and [illegible]0 ml of absolute aldehyde free EtOH using Norite Melting point 90.5 91.0 C. A mixture of 1% solution with equal portions of aqueous [illegible] N NaOH gives no color after 60 minutes A 2% solution in absolute aldehyde free EtOH stored in the dark may be used for 10-14 days.
4. Hydrochloric acid (HCl) concentrated
5. Carbon tetrachloride (CCl_4) C.P.
6. Ethyl alcohol (C_2H_5OH) absolute
7. Sodium hydroxide (NaOH) approximately [illegible] N Dissolve 80 gm in 1000 ml water

Quaife M L, and Harris P L The Chemical Estimation of Tocopherols in Blood Plasma, J Biol Chem 156 (2) 499 505 (Dec) 1944
Quaife, M L, Scrimshaw, N S and Lowry O H A Micromethod for Assay of Total Tocopherols in Blood Serum J Biol Chem 180 1229 1235 (Oct) 1949

Trigonelline

Kodicek E, and Wang, Y L *A New Method for the Estimation of Trigonelline in* Urine and Food tuffs, Nature 148 23 24 (July) 1941

Vitamin A and Carotene

Adlersberg D, Kann, S, Maurer A P Neverly K, Winternitz, W, and Sobotka H Studies on Serum Carotene in Man Am. *J Digest D s 16 (9)* 333 337 (Sept) *1949*
Clausen S W and McCoord A B The Carotinoids and Vitamin A in the Blood, J *Pediat* 13 (5) 635 650 (Nov) 1938
Kawasaki C, and Suenaga S Colorimetric Estimation of Vitamin A by Glycerodichlorohydrin Reagent J Pharm Soc Japan 69 460-462 1949
Koch W and Kaplan, D A Simultaneous Carr Price Reaction for the Determination of Vitamin A J Biol Chem 173 (1) 363 369 (March) 1948
Thompson S Y *A Photoelectric Spectrophotometer Suitable for the Measurement of* Vitamin A by the Antimony Trichloride Reaction British J Nut 3 (1) 43 50, 1949

Vitamin D

DeWitt, J B and Sullivan M X Spectrophotometric Procedure for Quantitative Estimation of Vitamin D Ind. Eng Chem (Anal Ed) 18 117 119 (Feb) *1946*
Muller P B The Colorimetric Determination of Vitamins A D and β Carotene With *Particular Regard to Vitamin D Helv Chim Acta* 30 (5) 1172 1190 1949
Sobel A E, Mayer A M and Kramer B A New Colorimetric Reaction of Vitamin D_2 and D and Their Provitamin Ind Eng Chem. (Anal Ed) 17 (3) 160 165 (March) 1945

SECTION IV

BIOCHEMICAL PROCEDURES (Continued)

F HORMONES

1 Ketosteroids in Urine

Reference

Consolazio W V and Talbott J H The Extract on and Determination of 17 Ketostero ds in Urine J Endocrinol 27 355 9 (Sept) 1940

Principle

The ste o d linked to glucuronic ac d is set free by hydrolyzing with hydrochloric ac 1 and is extracted with carbon tet achloride Inte fering substances are washed out and the result ng extract s equilibrated with alkaline meta dinitrobenzene The final color is measured

Apparatus

1 One continuous extractor unit (See reference Made by MacAlaster Bicknell Company Camb dge Mass See Fig 3)
2 Steam hydrolyzer (According to Consolazio and Talbott)
3 All glass st ll to fit round bottom extraction flask
4 Automatic syringe p pette 0 2 ml capacity
5 S x pear shaped separatory funnels (250 ml capac ty) with interchangeable ground glass stoppers
6 Swivel separatory funnel rack
7 Coleman Jr Spectrophotometer
8 A quant ty of 19 x 150 mm cuvettes
9 A quantity of unglazed porcelain
10 A water bath set at 25 C
11 An electric centrifuge
12 A steam bath

Reagents

1 Potassium hydroxide (KOH) exactly 2 N
Ethyl alcohol (EtOH) aldehyde free
3 Meta din trobenzene $C_6H_4(NO_2)_2$ 2% Dissolve 20 gm in 750 ml of EtOH Heat to 40 C and add 100 ml of N NaOH After 5 minutes cool and add 2500 ml of water Filter and wash thoroughly with water Dry by suction Re crystallize twice from 120 ml and 80 ml of absolute aldehyde free EtOH us ng Nor te Melt ng point 90 5 91 0 C A mixture of 1% solution w th equal por t ons of aqueous 2 N NaOH gives no color after 60 minutes A 2% solution in absolute aldehyde f ee EtOH stored n the dark may be used for 10 14 days
4 Hydrochlor c ac d (HCl) concentrated
5 Carbon tetrachloride (CCl_4) C P
6 Ethyl alcohol (C_2H_5OH) absolute
7 Sodium hydrox de (NaOH) app ox mately 2 N D ssolve 80 gm in 1000 ml water

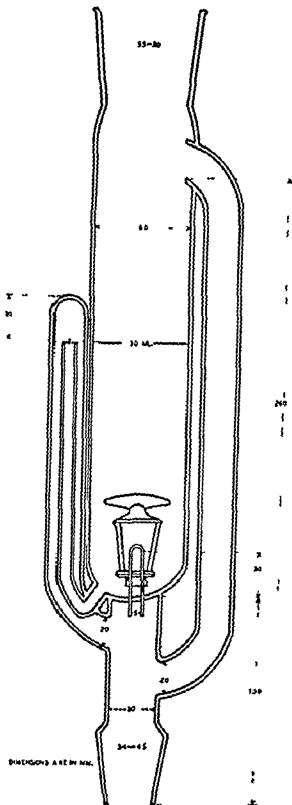


Fig 23.—Extraction unit (Reproduced by permission of the manufacturers)

8 Sodium hydrosulfite (Lycopon) (Obtained from Central Scientific Company) To 100 ml of 1 N NaOH add 10 gm of Lycopon Mix quickly pour into a separatory funnel and cover with light mineral oil
9 And osterone standard. Dissolve 250 mg androsterone in 1000 ml aldehyde free absolute EtOH One ml = 0 5 mg
10 Mineral oil
11 Norite

Collection and Storage of Urine

Collect 2 overnight specimens on successive n ghts keeping the specimens cold unt l brought to the laboratory Use no preservative Pool the two specimens and if they measure not more than 1000 ml extract the total pool No sample should be assayed which shows signs of putrefaction If necessary to keep the urine ac dify it and keep it in a refrigerator

Procedure

1 Into a steam hydrolyzer measure 500 ml of urine and 50 ml of concent ated HCl *Hydrolyze with steam for 0 minutes*
3 Cool with running water
4 Add app oximately 50 ml of CCl to extractor and approximately 75 ml of CCl to a round bottomed flask
5 Run hydrolyzed urine into extracto make proper connections and extract for 60 m nutes
6 Into 4 separatory funnels pipette 10 ml of 2 N sodium hyd oxide
7 Suck CCl_4 extract into first funnel and wash extract flask with two 10 ml portions of CCl
8 Wash 17 ketosteroids (a) 4 times with 10 ml of 2 N sodium hydroxide (b) 1 time with 10 ml of 10% Lycopon in 1 N NaOH until the pigment disappears Shake for 30 seconds longer Pigments disappear when all oxygen has been absorbed *Do not open* separatory funnel until compl te separation has occurred *Then deliver rapidly into a separatory funnel containing 5 50 ml of water* If this step is not carried out as described the red pigment appears again (c) Wash with water
9 After washing with water let settle for 10 minutes and deliver directly into dist lling flask Add a piece of unglazed porcelain
10 D still off CCl
11 Dry thoroughly w th suction and dilute to 5 0 ml or 10 0 ml with aldehyde free EtOH
1 Into dry centrifuge tube p pette 0 ? ml of alcohol c extract
13 Add 0 . ml of 2% meta d nitrobenzene
14 Add 0 ml of N potassium hydroxide Stopper with new clean cork
15 Equilibrate for 5 minutes at 25 C
16 Add 10 0 ml absolute EtOH Mix.
17 Centrifuge *Transfer to a cuvette (Caut on Centrifuge must be cold.)*
19 Read within 15 minutes at 5 0 mμ setting the blank at 100% T

Standard Curve

1 *0 2 ml of androste one in EtOH.*
2 0 ? ml of meta dinitrobenzene in alcohol
3 0 ml of 2 N KOH
4 *Equ librate 5 minutes at 5 C*
5 Add 10 0 ml of absolute EtOH (*If androsterone is dissolved in more than 0 2 ml of EtOH add androsterone to tube and evaporate EtOH with air current Then add 0 ml aldehyde free EtOH 0 ml meta dinitrobenzene and proceed*) The points in the standard curve range from 0 05 mg through 0 .5 mg of and osterone in the cuvette

Calculation

$$\text{mg 17 Ketosteroids/1000 ml} = \frac{(\text{mcg in cuvette}) \times (\text{total ml extract}) \times 1000}{1000 \times (\text{ml aliquot}) \times (\text{ml urine sample})}$$

Example

Volume of urine extracted 500 ml Volume of extract 10 ml Volume of extract assayed 0 . ml Reading ".%T equivalent to 180 mcg in cuvette

$$\text{mg 17 Ketosteroids/1000 ml urine} = \frac{180 \times 10 \times 1000}{1000 \times 0. \times 500} = 18 \text{ mg/1000 ml}$$

Precautions

1 All glassware must be chemically clean especially after separation of urine The glassware used after the ether phase must be dry A useful additional precaution is to heat glassware in the oven to destroy any steroids Stop-cock grease should not come in contact with solutions if possible It is necessary to grease stopcocks each time they are used

2 Cork stoppers must never come in contact with solutions Use tinfoil over them or use ground glass junctions

2 Hormones Miscellaneous References

Acetylcholine

Tower D B and McEachern, D Experiences With the 'Venus Heart Method for Determining Acetylcholine Canad. J Research 26B 183 187 (April) 1948

Wait R B The Action of Acetylcholine on the Isolated Heart of Venus Mercenaria Biol Bull 85 79 85 (Aug) 1943

Adrenaline

Annersten S Gronwall A and Koiw E The Fluorimetric Determination of Adrenaline in Blood Plasma Scandinav J Clin & Lab Invest 1 60 69 1949

Bloor W P and Bullen S S The Determination of Adrenalin in Blood J Biol Chem 138 (2) 727 739 (April) 1941

Jorgensen, K S Determination of Adrenaline Content of Blood With the Fluorescence Method Acta Pharmacol Toxicol 1 .7 739 1941

Kobro M Investigations of Adrenalin Concentration in the Blood I A Quantitative Chemical Method for Determination of the Concentration of Adrenalin in the Blood Acta med. Scandinav 124 511 5?1 1946

Whitehorn J C A Chemical Method for Estimating Epinephrine in Blood J Biol Chem 108 (3) 633 643 (March) 1935

Androgens

Callow N H Callow R K and Emmens C W Colorimetric Determination of Substances Containing the Grouping CH CO in Urine Extracts as an Indication of Androgen Content, Bioch m J 32 II 1312 1331 (June) 1938

Hilmer P E and Hess W C Spectrophotometric Determination of Androsterone and Testosterone, Anal Chem 21 8?. 8?3 (July) 1949

Langstroth G O Talbot N B, and Fineman A Spectrochemical Assay of Androsterone and Dehydroisoandrosterone in Simple Solutions J Biol Chem 130 (2) 585 591 (Oct) 1939

Munson P J Jones M E McCall P J and Gallagher T F A Colorimetric Method for Estimations of Dehydroisoandrosterone and Its Applications to Urine Extracts J Biol Chem 176 ?3 8? (Oct) 1948

Nielsen, A T Colorimetric Assay of Testosterone, Acta Endocrinol 1 36? 374 194?

Cortical Steroids

Schneider J J Studies on the Excretion of Adrenocortical Compounds I Isolation of the 17 Hydroxy 11 Dehydrocorticosterone in Normal Males J Biol Chem 183 363 376 (March) 1950

Corticosteroids

Corcoran A C and Page I H Methods for the Chemical Determination of Corticosteroids in Urine and Plasma J Lab & Clin Med. 33 1? 6 1333 (Oct) 1948

Daughaday W H Jaffe H and Williams R H Chemical Assay for 'Cortin Determination of Formaldehyde Liberated on Oxidation With Periodic Acid J Clin Endocrinol 8 166 174 (Feb) 1948

Furchgott R F Rosenkrantz H and Shorr E Infra red Absorption Spectra of Steroids IV Adrenal Cortical Hormones and Related Steroids J B ol Chem 171 ( ) 523 5 9 (Dec) 1947
Talbot N B Saltzman A H W xom R L and Wolfe J K The Color metric Assay of Urinary Corticosteroid like Substances J Biol Chem 160 ( ) 535 546 (Oct) 1945
Venning E H Kazmin V E and Bell J C B olog cal Assay of Adrenal Corticoids Endocrinol 38 ( ) 9 89 (Feb) 1946

Estrogens

Cohen H and Bates R W A Simple Quantitative Colorimetric Method for Estrogenic Steroids J Clin Endocrinol 7 (10) 701 707 (Oct) 1947
Dunford R A The Colorimetric Determination of Some Synthetic Oestrogens Canad J Research 7 B 646 65 (July) 1949
Evans J S Varney R F and Koch F C The Mouse Uterine Weight Method for the Assay of Estrogens Endocrinol 28 ,47 752 (May) 1941
Finkelstein M Microdete mination of Stero d Estrogens in Urine by Fluorom try Proc Soc Exper Biol & Med. 69 181 184 (Nov Dec) 1948
Jailer J W A Fluorometr c Method for the Determination of Estrogens Endocrinol 41 198 01 (Aug) 1947
Malpress F H The Colorimetric Estimation of St lboestrol Hexoestrol and Their Glu curon des n Ur ne B och m J 43 13 1 6 1948
Stevenson M F and Marr an G F The Determination of Oestrogens in Human Preg nancy Ur ne A New M thod of Correcting for the Brown Colour Developed in the Kober Reaction by Non oestrogenic Substances B ochem J 41 (4) 507 511 194,
S ego C M and Roberts S The Determination of Protein Bound Blood Estrogen Endoc nol 41 3 3 4 (Oct) 1947
Tubis M and Bloom A Color metr c Determination of D ethylst lbestrol Ind Eng Chem (Anal Ed) 14 309 194
Van Bruggen J T A Comparison of Methods Used for the Hyd olysis of Conjugated Urina y Estrogens J Lab & Cl n Med 33 0, 215 (Feb) 1948

Gonadotrophins

Dekanski J The Kaolin Adsorpt on Method for the Quantitat ve Assay of Ur nary Gonadotroph ns Brit J Exper Path. 30 (4) 97 3 (Aug) 1949
Hask ns A L Jr and Sherman A I Quant tative Bio Assay of Chorionic Gonadotrophin With the Male Frog Endocr ol 44 54 545 (June) 1949
Sm th I H Albright F and Dodge E Modifications in Method for the Precipitation and Assay of Inc eased Amounts of Pituitary Gonadotropic Substances in the Urine J Lab & Cl n Med 28 1761 1766 (Nov) 1943
Varney R F and Koch F C A Method for the Assay of the Gonadotropin Content of Normal Human Ur ne Endocrinology 30 399 407 (March) 1942

Histamine

M Intire F C Roth L W and Shaw J L The Pu ification of Histam ne for Bio assay J B ol Chem 170 (2) 53 544 (Oct) 1947

Parathormone

Opieńska Blauth J Preparat on and Dete minat on of Pa athyroid Hormones Ann Un v Maria Curie Sklo lowska Lublin Polonia Sec D 2 13 38 1947

Pituitary Adrenocorticotropin

Fo sham P H Thorn G W Prunty F T G and H lls A G Cl n cal Stud es With Pitu ta y Adrenocorticotrop n J Cl n Endocrinol 8 15 66 (Jan) 1948
Locke W Albert A and Kepler E J Adrenot ophic Activ ty of Human Urine Proc Soc Expe Biol & Med 72 ( ) 470 474 (Nov) 1949
Sayers M A Sayers G and Woodbury L A The A say of Adrenocort cotroph c Hor mone by the Ad enal As orbic Ac d Depletion Metho l Endocr nol 42 379 393 (May) 1948

Protein Bound Iodine

Ba sett A M Coons A H Salter W T and S mmons S M Protein Bound Iodine in Blood V Naturally Occurring Iodine F actions and Their Chemical Behavior Am J M Sc 202 516 5 1941

Steroids

Barnett J Henly A A and Mo s C J O R The Polarographic Estimat on of Steroid Hormones I Polarography of Neutral 17 Ketosteroids in Ur na y Extracts Biochem J 40 445 449 1946

Clark L C Jr and Thompson H A New Series of Reagents for the Colorimetric Determination of Steroids Science 107 4[illegible]9 431 (April) 1948
Dobriner K Lieberman S and Rhoads C P Studies in Steroid Metabolism I. Methods for the Isolation and Qualitative Estimation of Neutral Steroids Present in Human Urine J Biol Chem 172 [illegible]41 261 (Jan) 1948
Drekter I J Pearson S, Bartczak E and McGavack T H A Rapid Method for the Determination of Total Urinary 17 Ketosteroids J Clin Endocrinol 7 (1) 795 800 (Dec) 1947
Engel L L Patterson H R Wilson H and Schinkel M The Colorimetric Estimation of Urinary Neutral Steroid Alcohols J Biol Chem. 183 47 72 (March) 1950
Fraser R W, Forbes A P Albright F Sulkowitch H, and Reifenstein E Jr Colorimetric Assay of 17 Ketosteroids in Urine J Clin Endocrinol 1 234 [illegible]6 (March 1941
Heard R D H and Sobel H VIII A Colorimetric Method for the Estimation of Reducing Steroids J Biol Chem 165 (2) 687 698 (Oct) 1946
Holtorff, A F and Koch F C The Colorimetric Determination of 17 Ketosteroids and Their Application to Urine Extracts J Biol Chem 135 377 [illegible] (Sept) 1940
Lieberman S Dobriner K, Hill B R Fieser L F and Rhoads C P Studies in Steroid Metabolism II Identification and Characterization of Ketosteroids Isolated From Urine of Healthy and Diseased Persons J Biol Chem 172 [illegible]63 [illegible]95 (Jan) 1948
Talbot N B Butler A M and McLachlan E The Colorimetric Assay of Total α and β 17 Ketosteroids in Extracts of Human Urine J Biol Chem 132 ([illegible]) 595 603 (Feb) 1940
Tompsett S L and Oastler E G The Excretion of Corticosteroids The Determination of the Total Free Reducing Ketosteroids in Urine Glasgow M J 28 349 365 (Nov) 1947
Tompsett S L The Determination of Steroids in Urine The Analyst 74 6 17 (Jan.) 1949
Wilson H and Carter P Stabilization of the Alcoholic Potassium Hydroxide in Colorimetric 17 Ketosteroids Determinations Endocrinol 41 (5) 417 4[illegible]1 (Nov) 1947

Thyroxine

Pittrivers R and Lerman J Physiological Activity of the Optically Active Isomers of Thyroxine J Endocrinol 5 (5) 2[illegible]3 [illegible] 8 (June) 1948

SECTION IV

BIOCHEMICAL PROCEDURES (Continued)

G ENZYMES

1 Lipase in Serum

Reference

Johnson T A and Bockus H L Diagnostic Significance of Determinations of Serum L pa e Arch Int Med 66 6 7 (July) 1940

Principle

This method depends on the estimation of fatty acids prod ced by the hydrolysis of an ol ve oil emulsion on wh h lipase in the blood serum has been acting dur ng a 4 hou period und r optimum condit ons of pH and temperature

Apparatus

1 A water bath set at 100 C
2 An incubator set at 3 5 C
3 Accu ately calib ated 0 ml and 1 ml syringe pipette
4 A 10 ml burette
5 A Waring Blendor

Reagents

1 Phenolphthalein solut on 1% in alcohol
2 Disod um hydrogen phosphate (Na_2HPO H O) Dissolve 59 35 gm in 1000 ml of d stilled water to make an M/3 solution
3 Potassium dihydrogen phosphate (KH PO) Dissolve 45 38 gm in 1000 ml of dis tilled water to make M/3 solution
4 Mixed buffer solut on Mix 10 parts of Na_2HPO H O with 3 parts of $KH PO_4$
5 Ol ve oil emulsion a 50% solution Prepare by using a 5% solution of acacia as the emuls fying agent Add 0 percent of sod um benzoate as a preservative M x in War ng Blendor
6 Ethyl alcohol 9 %
7 Sod um hydrox de 0 0 N See under volumetri analys s for making up 1 N sodium hydroxide and d lute exactly 0 tim s

Procedure

1 Pipette 3 ml of d stilled water into each of two 15 ml centrifuge tubes A and B
2 Add exactly 1 ml serum to each and shake
3 Place tube A (control) in a 100 C water bath for minutes to inactivate the lipase Be sure to cool before proceed ng
4 Add 0 5 ml of buffer solution to each tube
5 Then add ml of olive oil emuls on to each and shake
6 Incubate at 37 5 C for 4 hours
7 At the end of that t me add 3 ml of 9 % alcohol and 2 drops of phenolphthalein indicator
8 Tit ate each tube to a faint p nk color with 0 05 N sodium hydroxide

3 Cholinesterase in Serum and Cells

Reference

Michel H O Electrometric Method for the Determination of Red Blood Cell and Pla ma Cholinestera e Activity, Fed Proc 8 99 (March) 1949

Principle

Cholinestera e hydrolyses acetylcholine bromide with the production of acetic acid. The *resultant drop in pH is measured in a barbital phosphate buffer* when serum is mixed with a known exce s of acetylcholine bromide Results are expressed as decrea e in pH units per hour

Apparatus

1 Constant temperature bath at 25 C
2 Beckman pH meter Model G '
3 A number of 15 ml thin wall glass test tubes
4 Accurately calibrated 1 0 and 10 0 ml syringe pipettes
5 0 1 ml volumetric serology pipettes
6 Small glass beakers 20 ml capacity

Reagents

1 *Preparation of the Stock Buffer*

A stock buffer, which will keep approximately 1 month in a glass stoppered bottle at room temperature and which contains 0 1 M sodium diethylbarbiturate (Barbital Sodium Merck) and 0 0167 M K HPO *in distilled water is prepared as follows*

a 20 618 gm of barbital sodium and 2 9092 gm of K HPO are weighed accurately and placed in a clean one liter beaker About 50 ml of distilled water are added *This olution will have a pH of about 9 9 Dilute acid (approximately 0 1 N HCl, 1* ml of concentrated HCl to 120 ml of water) is added slowly and with constant stirring to bring the pH of the buffer down to between 8 2 and 8 4 If strong acid *is used if addition of the acid is rapid or if stirring is inadequate white crystals* of barbital may appear The e may be redissolved by heating and they will remain in solution if the solution is cooled slowly The buffer is then transferred with washing into a volumetric flask and brought to volume with distilled water

2 *Preparation of Working Buffer*

A working buffer must be made up fresh each day It is made from the stock buffer with a *salt solution containing* 1 5 N NaCl and 0 30 N MgSO in distilled water (87 68 gm NaCl and 18 06 gm of MgSO dissolved in water up to one liter) in the proportions 3 ml sto k buffer 10 ml salt solution and 87 ml distilled water This *gives 100 ml of* working buffer In the preparation of the working buffer exactly ml of stock buffer and 10 ml of salt solution are mixed with about 80 ml of distilled water The pH is then adjusted to exactly 8 00 by addition of approximately 0 1 N HCl if its pH is over 8 00 or approximately 0 1 N NaOH (4 gm NaOH per liter) if its pH is under 8 00 The buffer is then transferred with washing into a volumetric flask and brought to 100 ml with distilled water

3 *Preparation of Acetylcholine Solution*

A solution of acetylcholine bromide is u ed 3 73% in distilled ... The material as obtained commercially is impure and ... purified ... Commercially obtained acetylcho ... is dissol ... 95% alcohol in a glass stoppered flask This is ... cool in ... for hours ... Cold ether is then added until turb ... The ... placed in a refrigerator for 48 hours T ... separated by filtration through ... filt ... d t ... cold 95% alcohol and 0 50 ... and over NaOH ... will keep ... The 3 73%

Procedure

1 Pipette *10* ml *of* working buffer into a thin glass test tube and place in a water bath at 5 C for at least 10 minutes
2 Add 0 1 ml of unknown serum (or plasma) and 1 0 ml of acetylcholine solution to the buffer
3 The exact t me of addition of acetylcholine solution is noted. Mixing with a glass stirring rod is performed on the addition of the acetylcholine solution.
4 The test tube is stoppered (rubber) and placed back in the 25 C water bath
5 Readings are conveniently taken at exactly 1 hour but if this is impracticable they may be taken at any time after an hour and the decrease in pH corrected to one hour For sera w th very low cholinesterase values incubation for longer per ods than one hour (e g up to 5 hours) may be required to give reliable readings. The reading is noted and the corrected value for pH drop per hour determ ned from the accompanying table (Table 11) wh ch makes correction for non enzymat c hydrolysis of acetylcholine and change in rate of pH drop with varying pH s
6 It should be noted that all glassware must be scrupulously clean as small changes in pH may be caused by dirt and this will affect readings It is suggested that all readings be done in duplicate as a check and that with every set of unknown sera on wh ch determinations are being run a serum whose cholinesterase activity is known be run simultaneously as a check on the method

Calculation

1 Two significant corrections have to be made in the computat on of units Δ pH/hr
   a. Non enzymatic hydrolys s of the substrate
   b The effect of pH on the specific activity of cholinesterase
2 The complete calculation in corrected Δ pH units/hour =

$$\frac{(8\ 00 - \text{final pH} + \text{blank} \pm \text{specific effect of pH})}{(\text{Total hours})}$$

3 Table 11 incorporates the two necessary correct ons and in add t on the subtract on from pH 8 00

TABLE 11

MICHEL S METHOD FOR ESTIMATING CHOLINESTERASE ACTIVITY CORRECTED VALUES FOR pH DECREMENT PER HOUR FOR pH 6 0 TO pH 7 99

(This table incorporates into the observed Δ pH correct ons for nonenzymat c hydrolysis of acetylchol ne bromide and for effect of pH on specific activity and it subtracts the corrected pH from 8 00)

pH Units as Read	0 00	0 0	0 04	0 06	0 08
	Corrected Δ pH Units				
6	1 74	1 7	1 70	1 67	1 64
6 3	1 63	1 60	1 58	1 56	1 54
6 4	1 5	1 50	1 48	1 46	1 44
6 5	1 42	1 40	1 38	1 36	1 34
6 6	1 3	1 30	1 [illegible]	1 7	1 6
6 7	1 4	1 [illegible]	1 0	1 19	1 17
6 8	1 15	1 13	1 11	1 10	1 08
6 9	1 06	1 04	1 03	1 01	1 00
7 0	0 98	0 [illegible]	0 94	0 93	0 91
7 1	0 89	0 87	0 85	0 83	0 81
7 [illegible]	0 9	0 77	0 75	0 73	0 71
7 3	0 69	0 67	0 65	0 63	0 61
7 4	0 59	0 56	0 54	0 5[illegible]	0 50
7 5	0 47	0 45	0 43	0 40	0 38
6	0 36	0 34	0 3[illegible]	0 30	0 7
7 7	0 5	0 3	0 [illegible]0	0 18	0 16
7 8	0 14	0 1[illegible]	0 10	0 08	0 06
7 9	0 04	0 03	0 0	0 01	0 00

Chymotrypsin

Iselin B M Huang H T, and Niemann C A Colorimetric Method for the Determination of Chymotrypsin Activity, J Biol Chem 183 403 408 (April) 1950

Cytochrome Oxidase

Smith F, and Stotz, E A Colorimetric Method for the Determination of Cytochrome Oxidase, J Biol Chem 179 891 902 (June) 1949

Hyaluronidase

Kass E H, and Seastone C V Role of the Mucoid Polysaccharide (Hyaluronic Acid) in the Virulence of Group A Hemolytic Streptococci, J Exper Med 79 319 330 (March) 1944

Tolksdorf S McCready M H McCullagh, D R and Schwenk E The Turbidimetric Assay of Hyaluronidase J Lab & Clin Med 34 74 89 (Jan) 1949

Warren G H Durso J G and Levin N R A Modified Turbidimetric Method for the Assay of Hyaluronidase Endocrinology 43 48 51 (July) 1948

Dorfman A and Ott M L A Turbidimetric Method for the Assay of Hyaluronidase J Biol Chem 172 367 375 (Feb) 1948

Lipase

Seligman A L and Nachlas M M The Colorimetric Determination of Lipase and Esterase in Human Serum J Clin Investigation 29 (1) 31 36 (Jan) 1950

Lipoxidase

Sumner J B and Smith G N The Estimation of Lipoxidase Activity Arch Biochem 14 87 92 (July) 1947

Peptic Activity

Fodor P J Price V E and Greenstein J P Enzymatic Hydrolysis of Saturated and Unsaturated Tripeptides J Biol Chem 180 (1) 193 208 (Aug) 1949

Tomarelli, R M Charney J and Harding M L The Use of Azoalbumin as a Substrate in the Colorimetric Determination of Peptic and Tryptic Activity, J Lab & Clin Med 34 428 433 (March) 1949

Wesselman, H J and Hilty W W The Turbidimetric Determination of Peptic Activity, J Am Pharmaceut Assoc Sci Ed 37 357 359 (Sept) 1948

Peroxidase

Ettori, J The Estimation of Peroxidase Activity Biochem J 44 35 38 (Jan) 1949

Phosphatase

Andersch M A and Szczypinski A J Use of p Nitrophenylphosphate as the Substrate in Determination of Serum Acid Phosphatase Am J Clin Path 17 (7) 571 574 (July) 1947

Hudson P B Brendler H and Scott W W A Simple Method for the Determination of Serum Acid Phosphatase J Urol 58 (1) 89 92 (July) 1947

King E J and Armstrong A R A Convenient Method for Determining Serum and Bile Phosphatase Activity Canad M A J 31 (4) 376 381 (Oct) 1934

Sherlock S P V and Walshe V Hepatic Alkaline Phosphatase Histological and Micro Chemical Studies on Liver Tissue in Normal Subjects and in Liver and Bone Disease J Path & Bact 59 (4) 615 630 (Oct) 1947

Proteolytic Activity

Iselin B M and Niemann C A Procedure for the Determination of Proteolytic Activity J Biol Chem 182 821 8 8 (Feb) 1950

Schwartz T B and Engel F L A Photometric Ninhydrin Method for the Measurement of Proteolysis J Biol Chem 184 197 20 (May) 1950

Trypsin

Lineweaver H Fraenkel Conrat H and Bean, R S Determination of Trypsin in the Presence of Egg White Trypsin Inhibitor and Demonstration of Absence of Trypsin From Egg White J Biol Chem 177 205 207 (Jan) 1949

Spencer W H. Gastrointestinal Studies VIII A Method for the Quantitative Estimation of Trypsin in Gastric Contents J Biol Chem 21 165 167 (June) 1915

White F D and Bowman J M The Estimation of Trypsin Canad J Research 25 E 153 161 (Aug) 1947

SECTION IV

BIOCHEMICAL PROCEDURES (*Continued*)

H PIGMENTS

1 Hemoglobin (Sanford Sheard)

Reference

Sanford A H and Sheard C The Determination of Hemoglobin With the Photoelectrometer J Lab & Cl n Med 15 483 489 (Feb) 1930

Principle

Blood is diluted with enough water to insure that all of the hemoglobin is converted to oxyhemoglobin Complete solution is assisted by the addition of a small quantity of sod um carbonate

Apparatus

1 A Coleman Jr Spectrophotometer
Cuvettes 19 × 150 mm
3 Micro pipettes 0 1 ml accurately calibrated
4 A quantity of 50 ml volumetric flasks

Reagents

1 Sodium carbonate a 5% solut on in water

Procedure

1 Into a 50 ml volumetric flask containing approx mately 5 ml d stilled water p pette exactly 0 1 ml of blood r nsing the pipette out at least 6 t mes
Add 0 ° ml carbonate dilute to 50 ml m x transfer to cuvette set water blank at 100% T at 550 mμ and read unknowns

Calibration Curve

Determine the exact oxygen capac ty of blood specimens equil brated 0 30 minutes w th room a r us ng the Van Slyke techniq1e for analys s The values in volumes percent oxygen are first corrected for dissolved oxygen and then divided by a factor of 1 34 for convers on to grams of hemoglobin Set up d lutions of the blood using the Sanford Sheard method in the range 5 to ° gm hemoglobin/100 ml blood

Calculation

$$\text{gm hemoglobin/100 ml blood} = \frac{(\text{mg Hb/ml in cuvette}) \times (\text{total dilution}) \times 100}{1000 \times \text{ml blood}}$$

Example

ml blood 0 1 Total dilution 50 ml
mg Hb/ml in cuvette 0 30

$$\text{gm hemoglobin/100 ml blood} = \frac{(0\ 30) \times 50 \times 100}{1000 \times 0\ 1} = 15\ 0 \text{ gm/100 ml}$$

Precautions

1 The color is stable for at least 30 minutes at usual room temperatures, but is unstable in the heat
2 This method is good for clinical purposes For more precision and for fractionating the heme pigment fractions the method of Evelyn and Malloy is recommended.

Normal Hemoglobin Standards

Various authors have proposed different values for 'normal' or 100% hemoglobin For instance Haldane used 13 8 gm/100 ml and Halden 15 6 gm/100 ml The following distribution curve was derived from analyses of the oxygen capacity of 791 healthy males

OXYGEN CAPACITY (ml/100 ml BLOOD)	NUMBER OF SUBJECTS (% OF TOTAL)
From 15 6 to 17 0	1.2
From 17 1 to 18 0	3 9
From 18 1 to 19 0	15 4
From 19 1 to 20 0	5 3
From 20 1 to 21 0	32 6
From 21 1 to 22 0	16 2
From 22 1 to 23 0	4 6
From 23 1 to 23 6	0 8

The average of all analyses was 20 11 ml oxygen/100 ml or 15 0 gm Hb/100 ml

EXAMPLE OF HEMOGLOBIN CALIBRATION CHART

Direct conversion gm Hb/100 ml blood when 0 1 ml of blood is diluted to 50 ml

% T	0	1	2	3	4	5	6	7	8	9
	gm HB/100 ml BLOOD									
40	19 6	19 1	18 6	18 1	17 5	17 0	16 6	16 1	15 7	15 [illegible]
50	14 8	14 4	14 1	13 6	13 [illegible]	12 8	12 5	1[illegible] 0	11 6	11 3
60	10 9	10 6	10 1	9 8	9 5	9 2	8 8	8 4	8 [illegible]	7 9
70	7 6	7 3	6 9	6 6	6 4	6 1	5 8	5 5	5 [illegible]	4 9
80	4 7	4 5	4 3	3 9	3 6	3 4	3 3	3 1	[illegible]9	2 7

2 Total Hemoglobin (Evelyn Malloy)

Reference

Evelyn, K. A and Malloy H T (Modified) Microdetermination of Oxyhemoglobin Methemoglobin and Sulfhemoglobin in a Single Sample of Blood, J Biol Chem 126 655 662 (Dec) 19 8

Principle

Total hemoglobin is determined by converting all the hemoglobin present to cyanmethemoglobin and measuring the light absorption at 540 mμ

Apparatus

1 Coleman Jr Spectrophotometer Model 6
2 Micro pipettes 0 1 ml accurately calibrated
3 A variety of 50 ml volumetric flasks
4 Cuvettes 19 × 150 mm

Reagents

1 Phosphate buffer (pH 6 6) 0 1 M (See section on buffers)
2 Potassium ferricyanide a 20% solution in water
3 Sodium cyanide or potassium cyanide a 5% solution in water POISON!
4 Concentrated ammonium hydroxide

Procedure

1. Introduce exactly 0.1 ml of whole blood into a 50 ml volumetric flask containing approximately 25 ml of distilled water. Rinse pipette at least 6 times.
2. Add 10 ml of 1/10 M phosphate buffer pH 6.6 and mix.
3. Add 3 drops of 20% potassium ferricyanide, mix and let stand for 10 minutes.
4. Add 3 drops of 5% NaCN or KCN, mix and let stand 2 minutes.
5. Add 1 or 2 drops of concentrated NH_4OH and mix.
6. Dilute to 50 ml with distilled water.
7. Prepare a blank tube in same manner as above being careful to add exactly the same quantity of potassium ferricyanide.
8. Set blank at 100% T at 540 mμ and read the unknowns.

Calibration Curve

1. Determine the oxygen capacity of samples of blood as described under the method of Sanford and Sheard.
   Run appropriate dilutions through the Evelyn Malloy procedure in the range 5 to 25 gm hemoglobin/100 ml blood.

Calculation

$$\text{gm hemoglobin/100 ml blood} = \frac{(\text{mg/ml in cuvette}) \times (\text{dilution}) \times (100)}{(1000) \times (\text{ml blood sample})}$$

Example

62.5% T was equivalent to 0.30 mg Hb/ml in cuvette.
For analysis 0.1 ml blood was finally diluted to 50 ml.

$$\text{gm hemoglobin/100 ml blood} = \frac{(0\ \ 0) \times (50) \times (100)}{(1000) \times (0.1)} = 15.0 \text{ gm/100 ml}$$

Precautions

1. Be sure to wash out the micro pipette at least 6 times in the water.
2. The color is stable for at least 30 minutes.

Oxyhemoglobin, Methemoglobin, Sulfhemoglobin

Reference

Evelyn K A and Malloy H T Microdetermination of Oxyhemoglobin Methemoglobin and Sulfhemoglobin in a Single Sample of Blood J Biol Chem **126** 655 666 (Dec) 1938

Principle

The methemoglobin is measured photocolorimetrically after being converted to cyanmethemoglobin. The oxyhemoglobin is converted first to methemoglobin with ferricyanide and then to cyanmethemoglobin. Sulfhemoglobin is estimated by direct observation at 6 0 mμ with a correction for the absorption by oxyhemoglobin and cyanmethemoglobin at this wavelength.

Apparatus

1. Coleman Jr Spectrophotometer Model 6
2. Cuvettes 19 × 150 mm
3. Micro pipettes 0.1 ml accurately calibrated
4. Dropping bottles

Reagents

1. Phosphate buffer M/15 pH 6.6 (See Sørensen's tables in the section on buffers.)
   Phosphate buffer M/60 diluted from above when needed.
3. Potassium ferricyanide K F (CN) 20% ...

4 Sodium cyanide (NaCN), a 10% solution in water POISON!
5 Acetic acid (CH COOH) a 12% solution in water
6 Solution of 10% NaCN mixed within ONE HOUR of use with an equal volume of 12% acetic acid.
7 Ammonium hydroxide (NH OH) concentrated

Procedure for Methemoglobin

1 Pipette exactly 0 1 ml of fresh whole blood into 9 9 ml of M/60 pho phate buffer in cuvette Wash out the pipette at least 6 times
2 *Let stand for 2 minutes*
3 Make up a blank with 10 ml of M/60 buffer and set at 0 density at a wave length of 635 mμ.
4 Read the unknown ample (Reading D)
5 Add 1 drop of neutralized NaCN to convert MHb to MHbCN
6 After 2 minutes read again at 635 mμ with the same blank This is D_1. The *difference* $(D - D_1)$ *is proportional to the concentration of MHb*

Procedure for Sulfhemoglobin

1 Clear the solution for SHb determination by addition of 1 drop of concentrated ammonium hydroxide
2 Read in colorimeter at 620 mμ with water blank at 0 den ity This reading is D

Procedure for Total Hemoglobin

1 Pipette 2 ml of this solution into a second cuvette
2 Add 8 ml of M/15 phosphate buffer of pH 6 6 and 0 05 ml of 20% potassium ferricyanide
3 Let stand for 2 minutes to allow HbO to be converted to MHb
4 Add 1 drop of NaCN reagent to convert MHb to MHbCN for total hemoglobin
5 Read D_4 after 2 minutes at 540 mμ, with a blank consisting of 10 ml water 1 drop cyanide reagent and 0 05 ml 20% potassium ferricyanide

Calibration Curves

1 Estimate the total hemoglobin of samples of blood by means of the oxygen capacity as described under *the Sanford Sheard method for hemoglobin*
2 Following the procedure de cribed above run samples through the method for total hemoglobin and compute K_{Hb} the calibration constant for total hemoglobin
3 At the same time convert HbO to MHb as under total hemoglobin but run through as for methemoglobin and compute the calibration constant K_{MHb}
4 The calibration constant for sulfhemoglobin K_{SH} is assumed to b 10 unless a *sample of pure sulfhemoglobin can be prepared*

Calculation

1 $T = \text{gm total hemoglobin/100 ml blood} = \frac{100 \times D}{K_{Hb}}$

2 $M = \text{gm methemoglobin/100 ml blood} = \frac{100\ (D - D\ )}{K_{M\ H}}$

3 $S = \text{gm sulfhemoglobin/100 ml blood} = \frac{100 \times D - (8\ 5 \times M) - (4\ 4 \times T)}{10}$

4 Corrected value of T = T from equation 1 – 0 2 × S
5 HbO = Corrected T – M – S

Precautions

1 The least accurate part of this method is that for sulfhemoglobin Fortunately this compound is rarely present in amounts over 0 5 gm/100 ml blood.

4 Pigments Miscellaneous References

Carboxyhemoglobin

Klendshoj N C Feldste n M and Sprague A L The Spectrophotometric Determina tion of Carbon Monox de J Biol Chem 183 "9 303 (March) 1950

Chlorophyll

Goodwin R H Fluorometr c Method of Estimating Small Amounts of Chlorophyll a Anal Chem 19 789 /94 (Oct) 1947

Petering H G Benne E J and Morgal P W S mplification of the Petering Wolman Hibbard Method for Determ nation of Chlorophyll and Carotene Ind Eng Chem (Anal Ed) 13 236 (April) 1941

Chromoproteins

Drabk n D L The Distribution of the Chromoprote ns Hb Myoglobin and Cytochrome C in the T ssues of Different Species and the Relationship of the Total Content of Each Chromoprotein to Body Mass J Biol Chem 182 317 34 (Jan) 19.0

Cytochrome C

Prader A and Gonella A The Spectrophotometric Determination of Cytochrome C in Tissues Experient a 3 46.. 464 1947

Rosenthal O and Drabkin D L Spectrophotometric Studies XI. The Direct Micro Spectrophotometric Determination of Cytochrome C J Biol Chem 149 437 450 (Aug) 1943

Hemoglobin

Holden H F On the Colorimetric Determination of Haemoglobin Austral an J Exper Biol & M Sc 25 (1) 57 60 (March) 1947

Pennell R B Smith W E and Werkhe ser W C Preparation of Hemoglobin Solu tions Contain ng Hemoglobin Reducing Enzymes Proc Soc Exper Biol & Med. 65 (2) 35 96 (June) 1947

Peters J T A New Micro iron Method for the Determination of Hemoglobin in Blood From One Drop of Finger tip Blood South M J 40 (11) 9 4 9 6 (Nov) 1947

Porphyrins

Grinstein M and Wintrobe M M Spectrophotometric Micromethod for the Quanti tative Determination of the Free Erythrocyte Protoporphyrin J Biol Chem 172 (2) 459-467 (Feb) 1948

Nicholas R E H and Rimington C Qualitative Analysis of Porphyrins by Partition Ch omatography Scandinav J Clin & Lab Invest gation 1 1 18 1949

Sveinsson S L Rim ngton C and Barnes, H D Complete Porphyrin Analysis of Pathological Urines Scandinav J Cl n & Lab Invest gation 1 11 1949

Wat on C J Schwartz S and Hawkinson V Stud es of the Uroporphyrins II Further Stud es of the Porphyrins of the Urine Feces Bile and Liver in Ca es of Porphyria With Part cular Reference to a Waldenström Type Porphyrin Behaving as an Entity on the Tswett Column J Biol Chem 157 345 361 (Jan) 1945

BIOCHEMICAL PROCEDURES (*Continued*)
SECTION IV

I HYDROGEN ION CONCENTRATION

1 Indicators

Indicators are weak organic acids or bases whose salts are different in color from the acid They are buffers with buffer ranges characteristic for each indicator As the ratio of acid to salt form of the indicator changes during a titration a gradual change in color ensues, and the range of pH over which this change occurs determines what indicator may be used for any particular titration

When a strong mineral acid is titrated with a strong base almost any indicator may be used since the pH changes from acid to alkaline with a very small amount of alkali at the end point

When a weak organic acid such as acetic is titrated with a strong base the indicator must be one that changes color at alkaline pH This is because the weak organic acids are buffers and in order to titrate them completely, one must titrate well beyond their buffer range Phenolphthalein is often used for this kind of titration, but cannot be used in the presence of ammonium salts or of carbonic acid

When a weak base is titrated with a strong acid one must use an indicator whose color change is in the acid range Bromphenol blue is useful for these titrations

LaMotte American Aniline Company Eastman and Eimer and Amend supply either the dry indicator from which solutions can be prepared or standard solutions of indicators prepared according to Clark (See Table 13)

A useful universal indicator is prepared and used as follows

5 mg thymol blue
25 mg methyl red
60 mg bromthymol blue
60 mg phenolphthalein

Dissolve and make up to 100 ml of 95% ethyl alcohol Titrate with 0 01 N NaOH to a green color

The range of this solution is from pH 4 to pH 10 The color changes are

Red	pH 4	Green	pH 7
Orange	pH 5	Blue	pH 8
Yellow	pH 6	Indigo	pH 9
		Violet	pH 10

2 Buffer Solutions

Reference

Clark W Mansfield The Determination of Hydrogen Ions ed 3 Baltimore 1928 Williams and Wilkins Company

TABLF 13

INDICATOPS SELECTED BY CLARK AND I UBS (1917)
SUPPLEMENTED BY COHEN (19 7)

COMMON NAME	MOL WT	A	pK	RANGE	COLOR CHANGE ACID	COLOR CHANGE ALK.	B pH	C pH	ABSORPTION MAXIMUM ACID mμ	ABSORPTION MAXIMUM ALKALI mμ
Metacresol purple	38	6	1 1	1 2 2 8	red	yellow	conc HCl	6	533	
Thymol blue	466	21 5	1	1 ° 8	red	yellow	conc HCl	6	544	
Bromphenol blue	699	14 9	3 98	3 0 4 6	yellow	blue	0	7		59
Bromcresol green	6J8	14 3	4 67	3 8 5 4	yellow	blue	1	8		617
Chlorophenol	4 3	3 6	5 98	4 6 6 4	yellow	red		9		573
Bromphenol red	512	19 5	6 16	5 6 8	yellow	red	3	10		ˬ74
Bromcre ol purple	540	18 5	6 3	5 6 9	yellow	purple	3	10		ˬ91
Bromthymol blu	6 4	16 0	7 0	5 7 0	yellow	blue	4	10		617
Phenol red	3ˬ4	°3 2	7 9	6 8 8 4	yellow	red	5	11		58
Cresol ed	38	6 2	8 3	7 2 8 8	yello v	red	5	11		7
Metac e ol purple	38	26 °	8 3	7 4 9 0	yellow	purple	5	11		ˬ80
Thymol blue	466	21 5	8 9	8 0 9 6	yellow	blue	6	1		ˬ96
Cre ol phthalein			9 4	8 2 9 8	colorless	red	6	1°		

A = ml of 0 01 N NaOH required per 0 10 gm indicator to form mono sod um salt Dilute to 250 ml for 0 04% solution
B = approx. pH value of solut on requ red for full acid color
C = approx pH value of solution requ red for full alkal ne color

Clark W M The Determinati n of Hydrogen I ns d 3 B ltimo e 19_8 William & Wilkins Company

Definitions

1 A buffer is any substa ce or combination of substances which when dissolved in water produces a solution wh ch resi ts a change in its hydrogen ion concentra t on upon the addition of an acid or an alkali

2 pH = - log hydrogen ion concentration expressed n moles/liter (the pH of 0 01 N HCl in 0 09 N KCl at °0 C is taken as 2 038)

3 pK = pH at which a buffer is half ionized and half un onized.

Buffer Solutions

The various portions of Tables 14A to 14E give the directions for preparing buffer solutions of known hydrogen on concentration as recommended by different authors

3 Glass Electrodes

(Measurement of pH of Blood Plasma and Red Cell Under Anaerobic Conditions)

References

D ll D B Daly C A and Forbes W H pK of Serum and Red Cells J Biol Chem. 117 569 579 (Feb) 1937

Clark W M The Determ nation of Hydrogen Ions ed 3 Balt more 19°8 Williams and Wilkins Company p 10

Principle

Glass acts like a fluid in which hydrogen ions dissolve It is the efore poss ble to measure the boundary potentials when fluids of different hydrogen ion concentrations are on either s de of a th n glass membrane In the present method fluids may be handled anaerobically within a thin glass tube which a ts as the glass el ctrode

Apparatus

1 Glass electrode w th 0 10 N HCl AgCl half cell and water jacket for temperature control (See Fig 24) The electrode proper of Corning Glass No 015 is a

TABLE 14A

SØRENSEN'S PHOSPHATE MIXTURE FOR pH 5.8-8.2
(Biochem. Z 21 131 1909 and 22 352 1909)

1 M/15 Primary phosphate 9.08 gm KH_2PO_4/liter
2 M/15 Secondary phosphate 9.47 gm Na_2HPO_4 (anhyd)/liter

pH at 20 C	M/15 Na_2HPO_4 (ml)	M/15 KH_2PO_4 (ml)	Phosphate mEq/l
5.8	8.0	92.0	108.0
5.9	9.0	90.1	108.2
6.0	12.2	87.8	11[illegible]
6.1	15.3	84.7	115.3
6.2	18.6	81.4	118.6
6.3	22.4	77.6	122.4
6.4	26.7	73.3	126.7
6.5	31.8	68.2	131.8
6.6	37.5	62.5	137.5
6.7	43.5	56.5	143.5
6.809	49.6	50.4	149.6
6.862	52.5	47.5	152.5
6.909	55.4	44.6	155.4
6.958	58.2	41.8	158.2
7.005	61.1	38.9	161.1
7.057	63.9	36.1	163.9
7.103	66.6	33.4	166.6
7.154	69.2	30.8	169.2
7.212	72.0	28.0	172.0
7.261	74.4	25.6	174.4
7.313	76.8	23.2	176.8
7.364	78.9	21.1	178.9
7.412	80.8	19.2	180.8
7.462	82.5	17.5	182.5
7.504	84.1	15.9	184.1
7.561	85.7	14.3	185.7
7.610	87.0	13.0	187.0
7.659	88.2	11.8	188.2
7.705	89.4	10.6	189.4
7.754	90.5	9.5	190.5
7.806	91.5	8.5	191.5
7.909	93.2	6.8	193.2
8.018	94.7	5.3	194.7
8.10	95.8	4.2	195.8
8.20	97.0	3.0	197.0

U shaped tube with a wall less than 0.1 mm in thickness. With a length of 200 mm and inside diameter of 1.5 mm the volume is about 0.10 ml. The tube is bent by its own weight while a positive pressure is maintained within the tube and the mid portion heated gently with a small luminous flame. Then the wall of the tube is thinned by thoroughly cleaning and dipping in hydrofluoric acid. The process is controlled by frequently removing the electrode, rinsing in water and then observing the change in the amount of pressure required to bend the tube. A satisfactory electrode will give a potential of approximately 0.0615 volt per unit change in pH at 37° C. The electrode is held in position between the halves of a rubber stopper which is sealed and fastened together with thick rubber cement and a wire band. 0.1 N HCl surrounds the electrode in the inner chamber and fills the side tube F. An Ag AgCl electrode connected to an amplifier at E, is placed within a paraffined tube at D and a large glass bead is placed above the constriction in order to minimize movements of solution around the Ag AgCl electrode. Water from a constant temperature bath maintains the temperature by circulating in the outer chamber. We have used the electrode successfully at 0.5, 25, and 37° C. A small copper wire is grounded at G and passed

TABLE 14B

WALPOLE S ACETATE BUFFER MIXTURES FOR pH 3 6 TO 5 8
(J Chem Soc 105 2501 1914)

1 Sodium acetate 0 N 7 gm NaC H O 3H O diluted to 1000 ml with water
2 Acetic acid 0 N approximately 11 3 ml of 99% glacial acetic acid diluted to 1000 ml with water Standardize against 0 1 N sodium hydroxide with phenolphthalein as an indicator

pH	0 2 N Sodium acetate (ml)	0 2 N Acetic Acid (ml)
3 6	7 5	9? 5
3 8	1? 0	88 0
4 0	18 0	8? 0
4 2	?6 5	3 5
4 4	37 0	6? 0
4 6	48 0	52 0
4 8	59 0	41 0
5 0	70 0	30 0
5 ?	79 0	21 0
4	86 0	14 0
5 6	91 0	9 0
5 8	94 0	6 0

TABLE 14C

CLARK S 0 2 M BORATE BUFFER MIXTURES FOR pH 7 8 TO 10
(The Determination of Hydrogen Ions 19 8)

1 0 2 M boric acid 1 405 gm H_3BO_3/l
0 2 M potassium chloride 14 91 gm KCl/l
0 2 M sodium hydroxide from conc. CO free alkali

pH	0 M H BO 0 M KCl (ml)	0 2 M NaOH (ml)
7 8	50	6?
8 0	50	4 00
8 2	0	5 90
8 4	50	8 55
8 6	50	12 00
8 8	50	16 40
9 0	50	1 40
9	50	26 70
9 4	50	00
9 6	50	6 85
9 8	50	40 80
10 0	50	43 00

Dilute to 200 ml with distilled water in each case

through the rubber tubing and connection into the outer chamber in order to remove stray electric currents which might result from the circulating water

2 Calomel half cell with saturated KCl and KCl reservoir
3 Apparatus for amplification and measurement of potentials Any circuit which will measure accurately to 0 1 mv will serve
4 A constant temperature water bath set at 3? C
5 A number of 2 ml syringes

Reagents

1 Potassium chloride (KCl) a saturated solution in water
2 Sodium chloride (NaCl) isotonic 0 9 % solution in water
3 Buffers on each side of the range of the unknowns
4 For blood work use M/15 phosphate buffers *These should be kept in an ice* box as two separate solutions (a) and (b) and mixed as needed in the proportions shown in the accompanying Table 15

TABLE 14D

SØRENSEN S GLYCINE HYDROCHLORIC ACID MIXTURE FOR pH 1 1 3 5
(Ergeb Physiol. 12 393, 1912)

1 *Glycine sodium chloride solution 0 1 M*
Dissolve 7 505 gm of glycine and 5 85 gm of sodium chloride in 1000 ml of water
2 Hydrochloric acid an 0 1 N solution

Temperature = 18 C		
Glycine-NaCl (ml)	HCl (ml)	pH
0 0	*10 0*	*1 038*
1 0	9 0	1 146
2 0	8 0	1 251
3 0	7 0	1 419
4 0	6 0	1 645
5 0	5 0	1 932
6 0	4.0	2 279
7 0	3 0	2 607
8 0	2 0	2 9_2
9 0	1 0	3 341
9 5	0 5	3 679

TABLE 14E

SØRENSEN S GLYCINE SODIUM HYDROXIDE MIXTURES FOR pH 8 5 1° 5
(Ergeb Physiol. 12 393, 1912)

Reagents

1 Dissolve 7 505 gm of glycine and 5 85 gm of sodium chloride in 1000 ml of water (0 1 M glycine plus 0 1 M NaCl)
2 Sodium hydroxide an 0 1 N solution

Glycine (ml)	Sodium Hydroxide (ml)	18 C pH	₋0 C pH	30 C pH
9.5	0 5	8 58	8 53	8 3
9 0	1 0	8 93	8 88	8 67
8 0	2 0	9.36	9 31	9 08
7 0	3 0	9 71	9 66	9 42
6 0	4 0	10 14	10 09	9 83
5 5	4.5	10 48	10 4,	10 17
5 1	4 9	11 07	11 01	10 74
5 0	5 0	11 31	11 25	10 97
4 9	5 1	11 57	11.51	11 [illegible]
4 5	5 5	12 10	12 04	11 74
4 0	6 0	12 40	1° 33	1° 03
3 0	7 0	12 67	1° 60	1° 9
2 0	8 0	12 86	12 79	1° 47
1 0	9 0	12 97	1° 90	12 5,

(a) Monobasic potassium phosphate (KH_2PO_4) 9 078 gm/1000 ml
(b) Dibasic sodium phosphate (Na_2HPO), 9 469 gm/1000 ml
The salts must be dried at 95 100 C. Pure salts may be obtained from La Motte and Co

5 (a) For buffer of pH 6 21 to 37 C 5 ml of Na_2HPO_4 are mixed with 0 ml of *$KH PO_4$*
(b) For buffer of pH 7 35 at 37 C, 20 ml of Na_2HPO are mixed with 5 ml of KH_2PO

Procedure—Testing System

1 Rinse electrode twice with saline
2 Rinse twice with buffer
3 Put in sample of buffer

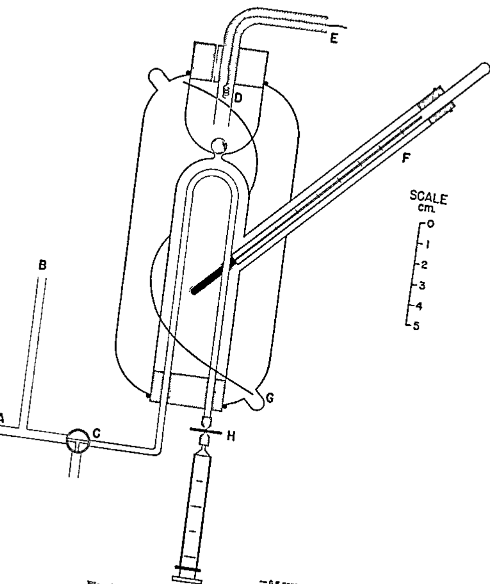


Fig 24 —Glass electrode f an e obic work.

TABLE 15

SØRENSEN S PHOSPHATE BUFFER SOLUTIONS AT 20 C AND 37 C FOR pH RANGE 5 8 TO 8.2
(M/15 acid pho phate 9 0 8 gm dry KH_2PO_4/liter,
M/15 basic pho phate 9 469 gm anhydrous Na_2HPO_4/liter)

pH		M/15 Phosphate Solution	
At 20 C	At 37 C	Na_2HPO_4 (ml)	KH_2PO_4 (ml)
5 80	5 77	8 0	9_0
5 90	5 87	9 9	90 1
6 00	5 97	12.2	87 8
6 10	6 07	15 3	84.7
6.20	6 17	18 6	81 4
6 24	6 21	20 0	80 0
6 30	6.27	2_ 4	77 6
6 40	6 37	26 7	,3.3
6 50	6 47	31 8	68_
6 60	6 57	37.5	6'5
6 ,0	6 67	43 5	56 5
6 80	6 77	49 6	50 4
6 90	6 87	55 4	44.6
7 00	6 97	61 1	38 9
7 10	7 07	66 6	33 4
7.20	7 17	72 0	28 0
7 30	7 27	76 8	23 2
, 38	7 35	80 0	_0 0
7 40	7 37	80 8	19 2
50	, 47	84 1	15 9
7 60	7._7	87 0	13 0
7 ,0	7 67	89 4	10 6
7 80	7 77	91.5	8 5
7 90	7 87	93 2	6 8
8 00	7 97	94 7	5 3
8 10	8 07	95 8	4 2
8.20	8 17	97 0	3 0

4. Flush stopcock with KCl.
5 Turn stopcock to connect KCl with buffer and read at once
6 Read again in one minute
   a. The two readings should be within 0 5 millivolts Temperature is probably drifting if greater divergence is noted. This drift must be eliminated. When a stable reading is obtained repeat steps 1 to 6 with second buffer Then repeat 3 6 with a second sample
   b These 4 readings on the second buffer should be within 1 mv of each other and should differ from the readings of the other buffer by 70 mv (i e 61.5 mv per pH unit at 37 C or 58 1 mv at 20 C) There is a linear relation between temperature and E M.F
   c If the difference between these two buffers (pH 6 81 and 7.35) exceeds 70 mv the buffers are not correct If it is less than 70 the buffers may be wrong but it is more probable that the trouble lies in the electrode It is either too thick, too old cracked or perhaps there is an electrical leak around it. However if the difference is constant and not less than 60 mv the electrode is usable.

Procedure—Measuring Unknown

1 Wash electrode four times with warm isotonic salt solution.
2 Put in blood sample by means of small syringe Sample should be fresh and must be handled as anaerobically as possible avoid getting oil into the electrode Push at least a quarter of a ml slowly through the electrode so that the blood in contact with the membrane is not the first blood layer to come from the syringe

3 Rinse stopcock with KCl
4 Turn stopcock to connect KCl with sample and read at once
5 Read again in 1 minute
6 Follow E M F for 5 minutes by occasional r adings There will probably be a greater change with the blood than with the buffers during the first minute but it should not be more than 1 mv The change between 1 and 5 minutes should be less than 1 mv Take the 1 minute reading
7 Repeat 2 3 4 and 5 if there is enough of the sample
8 Repeat steps 1 through 6 with the buffer which is nearest in pH to the unknown
9 Repeat steps through 6 with the other buffer If the temperature is constant and the electrode is working satisfactorily it is safe to use as many as 3 samples be tween rechecks against a buffer and the check aga nst the two buffers may be left to the end.

Calculation

1 pH of unknown =

pH acid buffer + [E M F unknown − E M F acid buffer] ÷

$$\left[\frac{\text{E M F alkaline buffer} - \text{E M F acid buffer}}{\text{pH alkaline buffer} - \text{pH acid buffer}}\right]$$

Example

pH alkal ne buffer	7 ?5
pH acid buffer	6 ?1
EMF alkaline buffer	3 0 1 mv
EMF ac d buffer	? 1 mv
EMF unknown	311 4 mv

$$\text{pH of unknown} = 6\ \ 1 + \left[(311\ 4 - \ 5\ \ 1) \div \frac{(3\ 0\ 1 - 25?\ 1)}{(7\ 35 - 6\ \ 1)}\right]$$

$$= 6\ ?1 + (59\ 3 \div 59\ 6)$$
$$= 7\ ?0$$

Precautions

1 All solutions put into the electrode should be within 1 or C of the temperature of the solution to be tested For blood this is 37 C Electrode must be constant within 0 1 and should be within 1 C of the temperature required It is not necessary to have the calomel cell and KCl at the same temperature as the electrode but they must not change in temperature during the experiment
2 Electrode should be left filled with water if in constant use and if not with 0 01 N HCl
3 Saturated KCl should not come in contact with electrode
4 The solutions are introduced with small syringes and should be at about the temperature of the electrode
5 The main difficulties with the glass electrode arise from the fragil ty of the electrode and its very high electrical resistance One is measuring a small po tential in series w th a resistance of several megohms and great care must be taken to avo d electrical leaks over moist surfaces. The N/10 HCl the KCl and the NaCl if spilt prom s uously are likely to lead to leaks especially in damp weather and if the electrode is used at low temperatures condensation will do the same
6 The temperature coefficient for drawn human blood under the cond tions of measurement s approximately 0 01 pH un ts per 1 C. As the temperature rises

the pH falls The same is true to a much smaller extent of the phosphate buffers However the E M F of the whole system changes greatly with temperature and one should work as near as possible to the desired temperature and be particularly *careful to avoid fluctuations*

4 Measurement of pH in Solutions
(Conditions Not Anaerobic Beckman pH Meter)

Principle

A glass electrode, when immersed in an unknown solution, develops a potential difference the magnitude depending on the hydrogen ion concentration of the solution This potential difference is measured by combining the glass electrode with some standard half cell such as the saturated calomel electrode and measuring the voltage of the system

Apparatus

1 A Beckman pH meter Model M

Reagents

1 Potassium chloride a saturated solution approximately 28 gm diluted up to 100 ml with water
2 Buffer solution pH 7 0 (see section on glass electrode under anaerobic conditions)

Procedure

1 Remove rubber cap from the end of the calomel electrode Mount the electrodes in their holder so that the calomel electrode projects slightly lower than the glass electrode and be sure they cannot touch the bottom of the beaker when the holder is lowered against the stop Remove the stopper from the calomel electrode during use
2 With control now in off position the meter should read exactly pH 7 0 Adjust if necessary by turning the screw below the meter s ale
3 With push button in lower right hand corner in up position, turn switch to on and leave in this position for a few seconds
4 Turn switch to 7 14 range and adjust meter to pH 7 with amplifier control knob in lower left corner
5 Turn switch to 0 7 range and adjust to pH 7 with standard knob Check the amplifier adjustment occasionally during use
6 Immerse electrodes in a buffer solution of pH 7 0
7 Place switch at proper range press button and adjust meter to buffer pH by means of the AP knob
8 To determine pH of test solutions clean electrodes immerse in test solution and press button The meter will indicate the pH automatically If the needle goes off the scale turn the switch to the other range

Calculation

1 pH is read directly

Precautions

1 The standard knob should require only infrequent adjustment
2 The electrodes should be immersed a full inch in the solution to avoid slight residual effects
3 When not in use the electrodes should be immersed in a beaker of water and the calomel electrode stopper replaced.
4 Keep the electrodes clean at all times and do not allow the test *solution to dry* on them

5 Keep the solutions at approximately °5 C as the apparatus has been calibrated at that temperature

6 When testing solutions of pH 9 , or higher containing alkali metal ions readings should be corrected for alkali ion error This correction may be determined experimentally

5 Hydrogen Ion Concentration, Miscellaneous References

Plasma pH

Van Slyke D D Weisiger J R and Van Slyke K K Photometric Measurement of Plasma pH J Biol Chem 179 743 756 (June) 1949

Urine pH

Van Slyke D D Weisiger J R and Van Slyke K K Photometric Measurement of Urine pH J Biol Chem. 179 7 7 761 (June) 1949

SECTION V

MICROBIOLOGICAL PROCEDURES

1 Pteroylglutamic Acid (Folic Acid)

Reference

Modified Microbiological Method of Tepley, L J and Elvehjem C A The Titrimetric Determination of Lactobacillus Casei Factor and Folic Acid J Biol Chem 157 303 309 (Jan) 1945

Principle

The response (acid production) of Lactobacillus casei E to graded increments of the sample and of a standard solution of pteroylglutamic acid added to media furnishing all the nutrients required by this microorganism except the nutrient under assay is measured by titration with sodium hydroxide The acid produced is a direct function of the original concentration of pteroylglutamic acid

Apparatus

1 Autoclave horizontal type
2 Beakers assorted 25 1000 ml
3 Burette 50 ml
4 *Centrifuge*
5 Cotton nonabsorbent
6 Graduat d cylinders glass stoppered assorted 25 1000 ml
7 Filter paper qualitative
8 Flasks Erlenmeyer 50 and 1°5 ml
9 Flasks volumetric glass stoppered assorted °5 2000 ml
10 Funnels 2 3 inch diameter
11 Incubator water jacketed electric (±0 ʋ C)
12 Inoculating needle platinum or nichrome steel
13 Automatic pipette (Fisher Volustat)
14 Graduated pipettes 0 5 1 0 2 0 5 0 10 0 and 25 0 ml
15 Volumetric transfer pipettes 0 5 1 0 2 0 5 0 10 0 2ʋ 0 and 50 0 ml.
16 Refrigerator
17 Arnold sterilizer
18 Hot air sterilizer
19 Sterilizing cans
20 Hypodermic syringes 5 to 10 ml
21 Interval timers
2" Test tubes uniform lipless Pyrex
23 Test tube covers aluminum rectangular
24 Test tube supports metal
25 Water bath constant temperature

Reagents

1 Water, glass distilled.
2 0 0" N NaOH and 0 1 N NaOH
3 0 1% bromthymol blue
4 1 N NaOH (approx.)

5 0 1 N HCl (approx)
6 Chloroform U S P
7 Toluene U S P
8 *Vitamin free acid hydrolyzed casein* 10%
9 *Adenine Guanine and Uracil* These are dissolved in 75 ml distilled water and 5 ml or more conc HCl Heat and dilute to 1 mg of each per ml of final solution
10 *Asparagine* Dissolve 10 gm *l* asparagine in 1 liter of water Add a few drops of chloroform and toluene
11 *Alanine* Dissolve 5 gm *dl* alanine in 50 ml of water Add a few drops of chloroform and toluene
12 *l Cystine and l Tryptophane* Each of these is dissolved in a small amount of 1 0 N HCl and diluted to 4 mg per ml and 2 mg per ml respectively
13 *Salt Solution A* Dissolve 25 gm KH_2PO_4 and 25 gm K_2HPO_4 in water to make 250 ml Keep under toluene and chloroform
14 *Salt Solution B* Dissolve 10 gm of $MgSO_4 \cdot 7H_2O$ 0 5 gm NaCl 0 5 gm $FeSO_4 \cdot 7H_2O$ and 0 4 gm $MnSO_4 \cdot H_2O$ in water and dilute to 250 ml Add 5 10 drops of HCl to clear solution Add chloroform and toluene
15 *Vitamin Solution* 10 mg of thiamin hydrochloride 60 mg pyridoxine hydrochloride 30 mg nicotinic acid 20 mg calcium pantothenate 0 5 mg p aminobenzoic acid and 1 ml salt solution A are added to 100 ml water Add chloroform and toluene and store in refrigerator
16 *Pteroylglutamic Acid Standard Solution* Weigh 10 mg pteroylglutamic acid and transfer to 1000 ml volumetric flask using 0 0 N NaOH to make up to volume Using 1 ml of this solution plus 1 ml of salt solution A dilute to 100 ml with 0 0 N NaOH for a standard stock solution of 100 mcg per ml Dilute to 0 1 milli micrograms per ml for the working standard
17 *Biotin* 25 mcg of crystalline (free acid) biotin are transferred to a 250 ml volumetric flask Add 7 ml of salt solution A and adjust to volume with water
18 *Riboflavin* Weigh 50 mg U S P reference standard riboflavin (dried in vacuum desiccator) and transfer to 2 liter volumetric flask Add 1500 ml H_2O 2 4 ml glacial acetic acid and warm Cool and make to volume This solution contains 25 mcg riboflavin per ml of 0 02 N acetic acid
19 *Yeast Dextrose Agar* 15 gm agar (Difco Bacto) are dissolved in about 150 ml of hot water After agar is in solution add 5 gm glucose 20 gm yeast extract and dilute to 1 liter Tube in 7 8 ml portions Plug tubes with nonabsorbent cotton and autoclave at 15 lbs pressure for 15 minutes Cool and store in refrigerator
20 *Yeast Dextrose Broth* Dissolve 5 gm Bacto yeast extract 10 gm $NaC_2H_3O_2 \cdot 3H_2O$ 5 gm glucose 5 ml salt A and 5 ml salt B in water Adjust to pH 6 8 7 0 and dilute to 1 liter Tube in 10 ml portions plug with nonabsorbent cotton and autoclave at 15 lbs pressure for 15 minutes Cool and store in refrigerator Maximum amount of pteroylglutamic acid used in preparation of standard curve may be incorporated with each tube as a supplement to the broth
21 *Isotonic Salt Solution.* Tube in 10 ml portions plug with cotton and sterilize in autoclave
22 *Lactobacillus Stock Culture* The test organism is a pure culture obtained originally from the American Type Culture Collections Georgetown University Medical School Washington D C as number 7469 The organism is carried as a stab culture in a yeast dextrose agar medium and transferred at weekly intervals
23 *Takadiastase* Dissolve 2 gm of commercial takadiastase in 100 ml of water

Procedure

1 *Preparation of the Inoculum* On the day prior to use transfer cells from the stock culture to a sterile tube of inoculum culture medium (Reagent 20) Incu

bate this culture for 16 24 hours at 37 5 C After incubation, the cells are centrifuged out, resuspended in saline and 1 drop added to 10 ml of 0 9 *per cent* saline For the inoculum one drop of this cell suspension is added per tube

2 *Preparation of Samples*

a Food Weigh accurately about 1 gm food Add 25 ml acetate buffer pH 4.5 1 ml takadiastase enzyme (2%) solution 2 ml of toluene plug with cotton and incubate 37 5 C for 18 24 hours Adjust pH to 6 6 6 8 steam for 10 minutes in autoclave dilute 1 2.0 for normal diet and filter

b Feces About 0 8 gm feces is treated by enzyme method above final dilution is 1 25 000

c Urine Dilute 2 ml urine to 100 ml with water in a dilution of 1 to .0

d Plasma and Blood Add 8 ml acetate buffer to 25 ml Erlenmeyer flask and add 0 1 ml blood or plasma Wash pipette several times with acetate buffer Add 0 1 ml 2% takadiasta e solution Gently mix and then add 1 drop of toluol to each Erlenmeyer flask *Always* run blank consisting of all components except sample Incubate at 37 5 C for 24 hours ± 2 hours Steam samples in autoclave for 15 minutes Cool and adjust to pH 6 6 6 8 Dilute blood 1 500, plasma 1 ?50

3 *Preparation of Basal Medium* Prepare the basal medium having the composition shown below About 500 ml is required for 100 tubes

20 gm anhydrous glucose
33 4 gm sodium acetate
1 25 gm K_2HPO
50 ml casein hydrolysate 10%
50 ml *l* tryptophane solution (2 mg per ml)
50 ml *l* cystine (4 mg per ml)
10 ml adenine guanine uracil (1 mg each per ml)
10 ml asparagine (10 mg per ml)
10 ml *dl* alpha alanine (20 mg per ml)
. ml salt B
4 ml vitamin solution

50 mcg thiamin	per ml
300 mcg pyridoxine	
150 mcg niacin	
100 mcg pantothenic acid	
2.5 mcg p aminobenzoic acid	
8 ml biotin 0 1 mcg per ml	
8 ml riboflavin ?5 mcg per ml	

Adjust pH to 6 6 6 8 and dilute to 500 ml

4 *Preparation of Standard Tubes* To duplicate tubes 13 × 100 mm, add 0 0, 0 15, 0 25 0 5 0 75 1 0 1 ?5 1 5, 1 75 and 2 0 ml of standard pteroylglutamic acid solution (0 1 millimicrogram per ml) Add sufficient water to bring the volume in each tube to 2 ml To each of these tubes add 1 ml of basal media An automatic pipetting machine is a valuable asset in this step

5 *Preparation of Assay Tubes* To duplicate tubes 13 × 100 mm add 0 5 1 0 1 5 and 2 0 ml of the specimen Add sufficient water to bring the volume to 2 0 ml. To each of these tubes add 1 ml of basal medium.

6 *Sterilization* Place aluminum covers over the assay tubes in racks Autoclave at 15 lbs pressure for 10 minutes

7 *Inoculation and Incubation* Cool all the tubes to the incubation temperature Aseptically inoculate each tube with one drop of inoculum. Incubat for 72 hours at 37 5 C

8 *Titration* Transfer the contents of a tube to a 50 ml Erlenmeyer flask rinsing once with 10 ml of water Titrate the contents of the flask with 0 0? N NaOH to a pH of 6 8 using bromthymol blue as an internal indicator

Calculation

1 Draw a standard curve for the assay by plotting millimicrograms of pteroylglutamic acid (abscissa) in the standard series against ml 0.0 N NaOH (ordinate)

2 Determine the pteroylglutamic acid content of the tubes in the unknown series by interpolation of the titer values on the standard curve Use only values which do not differ from the average by more than 10%

$$\text{millimicrograms folic acid/ml specimen} = \frac{\text{average/tube}}{\text{aliquot volume}} \times \text{dilution factor}$$

Example

The method of calculation is shown in the table below

ASSAY TUBE ml EXTRACT	TITRATION AFTER INCUBATION ml	PTEROYLGLUTAMIC ACID EVALUATED FROM STD CURVE mγ	PTEROYLGLUTAMIC ACID PER ml OF TEST EXTRACT mγ	
0.5	4.9	0.019	0.038	Av 0.031
1.0	5.9	0.030	0.0[illegible]0	
1.5	6.5	0.041	0.0[illegible]7	
2.0	8.0	0.068	0.034	

Dilution was 1 to 40 Final answer is therefore 40 × 0.031 = 1.24

Precautions

1 In order to avoid erratic results only glass distilled water and suitably pure research products should be used

2 Digestion with takadiastase and papain does not give maximum values for the folic acid content of many natural materials Each kind of material needs individual research to determine the best means for hydrolysis

2 Nicotinic Acid

References

1 Modified Microbiological Method of Snell E E and Wright L D A Microbiological Method for the Determination of Nicotinic Acid J Biol Chem 139 675 685 (May) 1941

Krehl W A Strong F M and Elvehjem C A Determination of Nicotinic Acid Ind Eng Chem Anal. Ed 15 471 475 (July) 1943

Principle

The microbiological determination of nicotinic acid is based upon the growth stimulation of Lactobacillus arabinosus In a basal medium that is niacin free but otherwise complete growth responses of the organism are measured quantitatively in standard and unknown solutions by titration of the acid produced

Apparatus

1 Same as in pteroylglutamic acid assay

2 *Vitamin Solution* Incorporate 5 mg thiamin hydrochloride 5 mg calcium pantothenate 5 mg p aminobenzoic acid 10 mg pyridoxine 1 ml salt solution A and dilute to 100 ml. Add chloroform and toluene and store in refrigerator

3 *Standard Nicotinic Acid Solution* A solution of nicotinic acid 0.5 mg per ml (500 micrograms per ml) is made by dissolving 1 15 mg of crystalline vitamin in the proper amount of distilled water 0 ml of this solution plus 1 ml salt solution A

are made up to 100 ml in a volumetric flask to give a concentration of 100 mcg per ml. Dilute to 50 millimicrograms per ml for working standard for microassay

4 *Stock Culture* Stock cultures are carried as stab cultures in a yeast dextrose agar medium. These cultures are transferred at weekly intervals incubated 24 hours at 37 5 C and then stored in the refrigerator until needed The organism used is Lactobacillus arabinosus 17 5 and may be secured from the American Type Culture Collection, where it is listed as No 8014

Procedure

1 *Preparation of the Inoculum* On the day prior to use transfer cells from the stock culture to a sterile tube of yeast dextrose broth supplemented with the maximum quantity of nicotinic acid used for preparation of standard curve For the inoculum the cells are centrifuged out the supernatant liquid decanted and the cells resuspended in 10 ml isotonic saline solution. One drop of this cell suspension is added to 10 ml of saline to provide a working cell suspension Use 1 drop per tube for inoculation

2 *Preparation of Samples*

a. Food—Weigh accurately about 1 gm food add 20 ml 1 N HCl autoclave 30 minutes at 15 lbs neutralize to pH 6 6 6 8 dilute 1 to 100 in water and filter

b Blood—Dilute 0 5 ml to 50 ml with water

c Urine—To 1 0 ml urine add 10 ml 1 N HCl autoclave 30 minutes at 121 C cool and adjust pH to 6 6 6 8 For assay dilute this solution 1 to 4 with water

d. Weigh accurately about 1 gm feces Add 20 ml 1 N HCl autoclave 30 minutes at 15 lbs Cool, adjust to pH 6 6 6 8 dilute to 100 ml with water and filter For use dilute filtrate 1 to 10 with water (i e final dilution 1 1000)

3 *Preparation of Basal Medium* Prepare a basal medium having the composition shown below

a Mix the following

20 gm anhydrous glucose
33 4 gm Na acetate $3H_2O$ (20 gm anhydrous)
50 ml casein hydrolysate 10%
50 ml tryptophane (2 mg *l* tryptophane per ml)
50 ml *l* cystine (4 mg per ml)
10 ml adenine guanine uracil (1 mg of each per ml)
2 ml vitamin solution (see reagents)
5 ml salt mixture A
5 ml salt mixture B
4 ml riboflavin (25 mcg per ml)
2 ml biotin (0 1 mcg per ml)

b *Adjust the pH to 6 6 6 8 Dilute to 500 ml with water*

4 *Preparation of Standard Tubes* Same as for pteroylglutamic acid assay using standard nicotinic acid solution (50 millimicrograms per ml) for working standard

5 *Preparation of Assay Tubes Sterilization Inoculation and Incubation Titration* These follow the same general microbiological techniques used in pteroylglutamic acid assay

Calculation

Same as for pteroylglutamic acid

Precautions

1 Lactobacillus arabinosus responds equally to niacin and its amide *Differential* microbiological assays have to be used to distinguish between the several metabolites of this vitamin.

3 Biotin

References

1 Modified Microbiolog cal Method of Shull G M Hutchings B L and Peterson W H. A Microbiological Assay for Biotin J Biol. Chem 142 913 9 0 (Feb) 194

2 Tepley L J and Elvehjem C A The Titrimetric Determination of Lacto bacillus Casei Factor and Folic Acid J Biol Chem 157 303 309 (Jan) 1945

Principle

B otin may be determined microbiologically by measurement of its effect upon the growth of Lactobac llus casei E The titratable acidity produced by L casei E is a function of the biotin concentration of the medium

Apparatus

1 Same as for pteroylglutamic acid assay

Reagents

Reagents are the same as for the assay of pteroylglutamic acid with a few ad ditions

1 Manganese dioxide (MnO U S P)
2 Nor te or activated charcoal
3 Superoxol (30% H_2O A C S)
4 Peptone (Difco Bacto)
5 Peptone Supplement Dissolve 5 gm of peptone n 100 ml of warm distilled water Adjust to pH 3 Stir w th °5 gm Nor te for 1 hour Filter Dilute to 00 ml Add 18 ml Superoxol (30% H O) Do not pipette!! Allow to stand at room temperature 24 hours Adjust to pH 7 Add about 15 gm MnO slowly with stirring When evolution of oxygen ceases filter solution and dilute to °50 ml with water Add chloroform and toluene and store in refrigerator

Stock Culture

Same as for pteroylglutamic acid

Procedure

1 *Preparation of the Inoculum* Same as for pteroylglutamic acid a say except in stead of pteroylglutamic acid the yeast dextrose broth should be supplemented with the minimum quantity of b otin used in preparation of the standard error

° *Preparation of Samples*

a Feces We gh accurately about 0 7 gm feces into a 50 ml Erlenmeyer flask Add 5 ml acetate buffer 1 ml °% papain (emuls fy 0 5 gm papain in 0 5 ml glycerine and dilute with 4 ml water) 1 ml 2°% taka diastase and 2 ml toluene Plug with cotton incubate 18 °4 hours at 37 C adjust to 6 6 6 8 steam 10 minutes and cool Dilute 1 to 100 with water filter and dilute the filtrate to a final dilution of 1 to 10 000

b Food We gh accurately a sample of about 1 gm Treat l ke feces through the stage of steaming Dilute 1 to 50 filter and use directly An alternative method is sometimes required add 1 gm food to 5 ml 1 N HCl autoclave 30 minutes at 15 lbs adjust to pH 6 6 6 8 d lute 1 o6 filter and use

c Urine Dilute one ml to 50 with water

d Blood and Plasma. Mix 1 0 ml blood or pla ma with 10 ml 1 N HCl autoclave at 15 lbs for 30 minutes and cool Adjust to pH 6 6 6 8 dilute 1 to 5 filter and use

Preparation of Basal Medium Prepare the ba al med um having the composition shown below

a Mix together

0 gm glucose

33 4 gm Na acetate 3H O (0 gm anhydrous)

1.25 gm K_2HPO_4
50 ml casein hydrolysate 10%
50 ml l tryptophane (2 mg per ml)
50 ml l-cystine (4 mg per ml)
10 ml adenine guanine uracil solution (1 mg each per ml)
10 ml asparagine (10 mg per ml)
10 ml dl alpha alanine (20 mg per ml)
10 ml peptone supplement (norite and peroxide treated)
4 0 ml vitamin solution (same as for pteroylglutamic acid)
1 0 ml folic acid (1 mcg per ml)
8 0 ml riboflavin (25 mcg per ml)
5 0 ml salt mixture B

b Adjust to pH 6 6 6 8 and dilute to 500 ml with water

4 *Preparation of Standard Tubes* Same as for pteroylglutamic acid assay using standard biotin solution (0 2 millimicrogram per ml) for working standard.

5 Preparation of assay tube sterilization inoculation and incubation titration and calculation are all according to the same general microbiological technics as used for pteroylglutamic acid assay

4 Pantothenic Acid

References

1. Modified Microbiological Method of Cheldelin V H. Hoag, E H and Sarett H. P The Pantothenic Acid Requirements of Lactic Acid Bacteria J Bact. 49 41-45 (Jan.) 1945
2. Hoag E H Sarett H. P and Cheldelin V H Use of *Lactobacillus Arabinosus* 17 5 for Microassay of Pantothenic Acid Ind. and Eng Chem Anal. Ed. 17 60 62 (Jan.) 1945

Principle

Pantothenic acid is estimated from its growth stimulation of Lactobacillus arabinosus 17 5 *Lactic acid formation is estimated titrimetrically The bound* vitamin is first liberated by means of digestive enzymes

Apparatus

1 Same as for pteroylglutamic acid assay

Reagents

Reagents are the same as for pteroylglutamic acid assay with a few additions.

1 *Alkali-Treated Peptone* To a solution of 50 gm peptone in 300 ml of distilled water is added 25 gm of NaOH also dissolved in 300 ml of distilled water The solution is placed under ultraviolet light and allowed to stand 24 hours The pH is adjusted to 6 6 6 8 with glacial acetic acid and 14 gm of sodium acetate are added and the volume made up to 1 liter This solution contains 5% peptones and 6% sodium acetate. Store in a refrigerator Toluene and chloroform are added.

2 *Vitamin Solution* Incorporate 5 mg thiamine hydrochloride 5 mg p-aminobenzoic acid 10 mg nicotinic acid 10 mg pyridoxine hydrochloride 1 ml of salt solution A and dilute to 100 ml. Add chloroform and toluene and store in refrigerator

3 *Standard Solution of Pantothenic Acid* A solution of calcium pantothenate 1 mg per ml, is made up by dissolving 10 20 mg of the crystalline vitamin in the proper amount of water Of this solution 5 4 ml plus 1 ml salt solution A are made up to 100 ml in a volumetric flask to give a concentration of 50 mcg per ml. Dilute to 10 millimicrograms per ml for the working standard.

4 *Stock Culture* The organism used is Lactobacillus arabinosus 17 5 (American Type Culture Collection No 8014) It is maintained on yeast agar stabs

Procedure

1 *Preparation of the Inoculum* Prepare the inoculum for the assay tubes by trans ferring from the stock culture to a sterile tube of yeast dextrose broth supple mented with .0 millimicrograms of calcium pantothenate Incubate for "4 hours at 37 5 C Centrifuge and discard supernatant liqu d Resuspend the cells in 10 ml of phys ological saline One drop of this cell suspension is added to 10 ml saline and one drop of this resuspended cell suspension is added per assay tube

2 *Preparation of Samples*

a. Food About 1 gm food is treated with 1 ml 2% takadiastase solution 25 ml acetate buffer pH 4 5 and " ml toluene Plug with cotton in ubate at 37 C for 18 24 hours Adjust pH to 6 6 8 0 steam 10 minutes in autoclave dilute and filter For use d lute 1 to 00

b Feces About 0 8 gm feces are treated with enzyme as above final dilution is 1 2000

c Whole Blood. 1 0 ml blood is treated as above final dilution is 1 40

d Plasma 1 0 ml plasma enzyme is treated as above final dilution is 1 20

e Urine Dilute 1 ml to "00 with water

3 *Preparation of Basal Medium* Prepare the basal med um having the composition below

a. Mix together

0 gm glucose
"" 6 gm sodium acetate
50 gm *l* tryptophane (2 mg per ml)
.0 ml *l* cystine (4 mg per ml)
100 ml peptone alkali treated (5% peptone 6% NaAc)
10 ml adenine guan ne uracil (1 mg each per ml)
50 ml casein hydrolysate 10%
5 ml salt solution A
5 ml salt solution B
3 ml vitamin solution (th amin and PABA 50 mcg per ml niacin and pyridoxine 100 mcg per ml)
3 ml biotin (0 1 mcg per ml)
8 ml r boflavin (2 mcg per ml)

b Adjust pH to 6 7 and d lute to 500 ml

4 *Preparation of Standard Tubes* Same as for pteroylglutam c acid assay using standard cal ium pantothenate solut on (10 millim crograms per ml) for the work ing standard

5 Preparation of as ay tubes sterilization inoculation and incubation t trat on and calculations are by the same general m crob ological techni s as were used in pteroylglutamic acid assay

Precautions

1 Lactobac llus arabinosus does not respond to bo ind pantoth n c ac d and since the vitamin is labile to heat in a d or alkaline solut on digestion of the sample must be accompl shed at neutral pH by means of d gest ve enzymes

5 Pyridoxine (Vitamin B_6)

Reference

Modified Microbiological Method of Atkin L Schultz A S Williams W L and Frey C N Yeast Microbiological Methods for Determination of Vitamins Ind and Eng Chem Anal Ed 15 141 144 (Feb) 1943

Principle

The growth stimulation of a strain of the yeast Saccharomyces carlsbergensis is used for assaying pyridoxine microbiologically Growth is estimated turbidimetrically

Apparatus

1 Same as for pteroylglutamic acid assay
2 Photoelectric spectrophotometer Coleman Jr
3 Mechanical shaker

Reagents

Same as for pteroylglutamic acid assay with some additions

1 *Sugar and Salt Solution* ?00 gm C P dextrose 2.2 gm monopotassium phosphate 1 7 gm potassium chloride 0 5 gm calcium chloride 0 5 gm magnesium sulfate 0 01 gm ferric chloride and 0 01 gm mangane e sulfate made up to 1 liter with water
2 *Potassium Citrate Buffer* 100 gm potassium citrate and 20 gm citric acid made up to one liter with water
3 *Casein Hydrolysate Solution* 10% Adjust the pH to 4 6
4 *Thiamine Solution* 10 micrograms per ml
5 *Inositol Solution* 1 mg per ml.
6 *Biotin Solution* 0 1 microgram per ml
7 *Calcium Pantothenate* ?00 micrograms per ml (0 2 gm per liter with 10 ml salt solution A per liter)
8 *Bacto Malt Extract* (Difco Bacto) and fresh moist baker s yeast
9 *0 44 N and 0.55 N H_2SO_4*
10 *Standard Pyridoxine Hydrochloride* A solution of pyridoxine hydrochloride 1 mg per ml is made up by dissolving 15 ?0 mg of crystalline vitamin in water 1 1 *ml of this solution plus 1 ml salt solution A are made up to 100 ml in a volumetric* flask to give a concentration of 100 mg of pyridoxine per ml. Two working standards are used 0 01 mcg per ml and 0 005 mcg per ml
11 *Stock Culture*
Saccharomyces carlsbergensis American *Type Culture Collection No 4??8, is carried* on Difco malt agar slants Fresh slants are incubated for 24 hours at 30 C and stored in a refrigerator for no more than two weeks

Procedure

1 *Preparation of the Yeast Inoculum* Prepare a fresh slant of Saccharomyces carlsbergensis on Difco malt agar and incubate 24 hours at 30 C Remove a quantity of fresh growth with a sterile wire loop and suspend in 10 ml of sterile isotonic *saline in a colorimeter tube* With the aid of a photoelectric colorimeter adjust the concentration of the yeast to an equivalent of 1 mg of moist bakers yeast per ml by adding sterile saline Dilute 10 ml of the adjusted suspension with 90 ml of saline in a sterile Erlenmeyer flask providing a yeast suspension with final con*centration of 0 1 mg per ml.*
2 *Preparation of Samples*
   a Food—Extract 1 gm of the specimen with 90 ml 0 44 N H SO by autoclaving at 15 pounds pressure for one hour Cool neutralize to pH 5 5 2, dilute to 100 ml and filter
   b Feces—Extract 0 5 gm of specimen with 90 ml 0 44 N H SO by autoclaving at 15 lbs pressure for one hour Cool neutralize to pH 5 5 2 dilute to 00 ml and filter
   c Urine—Extract 5 ml of specimen with 80 ml 0 05? N H SO by autoclaving at 15 lbs. pressure for 1 hour Cool neutralize at *pH 5 5 2 dilute to 100 ml* and filter
3 *Preparation of Basal Medium* Prepare the basal medium having the composition below
   a. Mix together

Sugar salt solution	250 ml
Potassium citrate buffer solution	50 ml

Casein hydrolysate	‿0 ml
Thiamine solution	5 ml
Inositol solution	"5 ml
Biotin solution	8 ml
Calcium pantothenate solution	12 5 ml or 50 ml P A. standard

b Dilute to 500 ml

4 *Preparation of Standard Tubes* Place ‿ ml of basal m dium in each of a series of 18 mm Pyrex test tubes Into duplicate tubes pipette 0 0 0 5 1 0 1 5 2 0 2 5 3 0 3 5 and 4 0 ml of diluted standard Adjust volume to 9 0 ml with water

5 *Preparation of Assay Tubes* Pipette into successive tubes 0 5 1 0 2 0 3 0 and 4 0 ml of the extract of spec men Adjust the volume to 4 0 ml and add ‿ 0 ml of the basal medium

6 *Sterilization* The tubes are covered with aluminum covers, steamed for 10 m nutes and cooled

7 *Inoculation and Incubation* Under aseptic conditions introduce into each tube 1 ml of the yeast inoculum Place the tubes in a mechani al shaker for 16 18 hours at 30 C (rate of about 100 strokes a minute)

8 *Estimation* Immediately after incubation estimate the y ast growth turbidimet rically in a photo lectri spectrophotometer Set the wave length at 680 mμ and 100% T with distilled water The yeast growth inoculum should read between 80 and 90% T

Calculation

1 On ordinary graph pap r plot the results of the reference series in per cent trans mittance against con entration of pyridoxine hydrochloride Estimate the values for the unknowns from the graph Calculate the pyridoxine content of the speci men for each tube and average all values which agree with n 10% of their mean fol owing the same general formula as for pteroylglutamic a id.

Precautions

1 The vitam n B group consists of pyridoxine pyridoxal and one or more labile factors All members of the complex show comparable activity for Saccharomyces carlsbergens s Hence this procedure is not specific for pyridoxine

2 Careful attention shoul l be given to the hydrolytic procedures employed to liber ate the bound forms of the vitamin it is extracted from most plant and animal tissues with d fficulty

3 The chief d sadva tage of this method is its low degree of precision On the average values may be regarded as reproducible to within 10% but frequently much greater variations are encountered for unexplained r asons

Riboflavin

References

1 Modified Microbiological Method of Snell E E and Strong F M. A Micro biological Assay for R boflavin Ind. and Eng Chem Anal Ed 11 346 350 (June) 1939

2 Scott M L Randall F E and Hess l F H. A Mod fication of the Snell and Strong M crobiological Method for Determining Riboflavin J Biol Chem 141 3 5 3 6 (Oct.) 1941

3 Strong F M and Carpenter L F Preparation of Samples for Microbiological Determination of Riboflavin Ind. and Eng Chem Anal. Ed 14 909 913 (Nov) 1942

Principle

Riboflavin is determined by measurement of the growth stimulation of Lacto bacillus casei E The acid p oduced by the m croorganism is determined by titra tion with sodium hydroxide

Apparatus

1 Same as pteroylglutamic acid assay

Reagents

Reagents are the same as for pteroylglutamic acid assay with a few additions

1 *Alkali Treated Peptone Solution* Dissolve 40 gm of peptone (Difco Bacto or Wilson s) in 250 ml of hot distilled water Add ⌐0 gm of NaOH in 250 ml of distilled water Mix the two solutions Allow to stand for 18 °4 hours then neutralize with glacial acetic acid Add 14 gm of dry NaC_2H_3O (or 3.2 gm $NaC_2H_3O \cdot 3H_2O$) and sufficient water to make up to 800 ml Preserve under chloroform and toluene in a refrigerator

2 *Yeast Supplement Solution* Dissolve 100 gm of yeast extract (Difco Bacto) in 500 ml of water and 150 gm of basic lead acetate in 500 ml of water Mix the two solutions Adjust the pH to 10 with concentrated NH_4OH and filter or centrifuge Adjust the filtrate to pH 6.5 with glacial acetic acid precipitate the excess lead with H_2S and add distilled water to make 1000 ml Preserve under toluene in the refrigerator

3 *Cystine Solution* Dissolve 1 gm *l*-cystine in 20 ml of 10% hydrochloric acid and dilute to 1000 ml with water Store under toluene in refrigerator not below 10 C

4. *Reference Standard Solution* Dissolve 50 mg of U S.P riboflavin reference standard in 1500 ml of distilled water containing ⌐4 ml of glacial acetic acid and warm to aid solution After cooling to room temperature make to 2000 ml volume with water *Preserve in the dark under toluene in a refrigerator* Dilute to 0 1 mcg of riboflavin per ml immediately before use for reference working standard.

Stock Culture

1 Same as for pteroylglutamic acid assay

Procedure

1 *Preparation of the Inoculum* On the day prior to use transfer cells from the stock culture to a sterile tube of yeast dextrose broth supplemented with 0 5 mcg of riboflavin/ml and incubate 24 hours at 37 5 C For the inoculum the cells are centrifuged out and the supernatant liquid decanted Suspend the cells in 10 ml of sterile isotonic saline solution and use 1 drop of this cell suspension.

2 *Preparation of Samples*

a. Foods To sample (10 or more micrograms riboflavin) add 50 ml 0 1 N HCl and autoclave for 15 minutes at 15 lbs pressure Add 1 ml of freshly prepared papain takadiastase solution (20 mg/ml) Incubate at 37 5 C for 4 hours. Heat with flowing steam for 10 minutes Adjust to pH 4.5 make to 100 ml volume and filter

b Urine Dilute with water so that the specimen contains between 0 05 and 0 5 mcg riboflavin per ml

3 *Preparation of Basal Medium* Prepare a basal medium having the composition below

a Mix together

Alkali treated peptone	50 ml
0 1% cystine solution	50 ml
Yeast supplement	5 ml
Salt solution A	⌐5 ml
Salt solution B	2 5 ml
Dextrose anhydrous	10 gm

b Adjust to pH 6 6 6 8 Dilute to ⌐50 ml

4 *Preparation of Standard Tubes* To duplicate tubes 16 by 150 mm in size add 0 0 0 5 1 0 1 5 2 0 3 0 and 5 0 ml of standard riboflavin solution (0 1 mcg per ml) Add sufficient water to bring the volume in each tube to 5 0 ml. To each of these tubes add 5 ml of basal medium

5 *Preparation of Assay Tubes* To duplicate tubes add 0 5 1 0 1 5 and 2 0 ml of the extract of the test material Add sufficient water to bring the volume in each tube to 5 0 ml and add 5 0 ml of basal medium

6 *Sterilization inoculation and incubation* are performed as in the pteroylglutamic acid assay

7 *Titration* Transfer the contents of each tube to an Erlenmeyer flask using 10 ml of distilled water for rinsing Titrate with 0 1 N NaOH using bromthymol blue as an indicator

Calculation

1 The same type of calculation is used as for pteroylglutamic acid assay

7 Tryptophane

References

1 Modified Microbiological Method of Snell E E and Wright L D A Microbiological Method for the Determination of Nicotinic Acid J Biol Chem 139 675 685 (June) 1941

2 Greene R D and Black A The Microbiological Assay of Tryptophane in Protein and Foods J Biol Chem 155 1 8 (Sept) 1944

3 Wooley J G and Sebrell W H Two Microbiological Methods for the Determination of *l* Tryptophane in Proteins and Other Complex Substances J Biol Chem 157 141 151 (Jan) 1945

Principle

The growth stimulation of Lactobacillus arabinosus 17 5 in relation to the tryptophane content of a basal medium is employed in the microbiological assay for this amino acid The lactic acid produced is titrated.

Apparatus

Same as for pteroylglutamic acid

Reagents

These are the same as for pteroylglutamic acid with some additions

1 *Standard Tryptophane Solution* Dissolve 0 40 mg of *l* tryptophane in the proper amount of hot water to provide a solution containing 2 mg tryptophane per ml In corporate 10 ml of the solution with 1 ml salt solution A and make up to 100 ml volume to give 200 mcg tryptophane per ml Dilute to 1 mcg per ml for working standard

Vitamin Solution Same as for pantothenic acid assay

3 *Stock Culture*

Same as for nicotinic acid assay

Procedure

1 *Preparation of Inoculum* Same as for nicotinic acid assay

2 *Preparation of Samples* Either of two methods may be employed

a. Use of Enzymes Place sample in 100 ml beaker (5 gm food 0 5 ml blood 0 5 ml plasma 1 ml urine) Add 5 ml 0 1 N H_2SO_4 0 5 ml 1% pepsin solution and incubate 18 24 hours at 37 5 C with occasional mixing Add 0 6 gm K_2HPO_4 and adjust pH to 8 4 Add 2 5 ml 1% trypsin solution 10 ml toluene and a few drops of chloroform. Incubate at 18 24 hours at 37 C in 1 5 ml Erlenmeyer flask with occasional mixing Adjust pH to 7 8 transfer to a 50 ml Erlenmeyer flask and add 5 ml 1% erepsin solution a few drops of chloroform and 10 ml toluene

Incubate 72 hours at 37 C with occasional mixing Replace toluene as necessary Adjust pH to 6 0 6 5, including blank, dilute to 200 ml and filter until clear using same filter paper for repeated filtrations Make final dilution as follows blood 1 to 3000 plasma 1 to 2000, diet 1 to 400, enzyme blank, 1 to 1000 on basis of pepsin urine 1 *to 20*

b Use of Barium Hydroxide Place sample containing about 250 mcg tryptophane in a 250 ml Erlenmeyer flask (diet 1 to 2 gm blood 0 1 ml, plasma 0 ml, urine 0 2 ml) Add 6 5 gm barium hydroxide Ba(OH) 8H O) and 0 ml water mix thoroughly and autoclave 16 hours at 15 lbs Autoclaved samples are transferred quantitatively while hot to 250 ml beakers After cooling the pH is made acid to 25 with 2 N H SO Samples are neutralized to pH 6 0 6 5 with NaOH and diluted to volume plus 1 ml to allow for BaSO formed Final dilutions are as follows food 1 to 50 or 1 to 150 depending on tryptophane content blood 1 to 1000 plasma 1 to 500 urine 1 to 100 Heat to 60 70 C filter and assay *Note* Because of racemization when barium hydroxide is used multiply all assay values by 2

3 *Preparation of Basal Medium* The basal medium recommended here differs from that of Snell and Wright s for niacin only in the omission of tryptophane and the inclusion of niacin

a *Mix together*

20 gm glucose
33 4 gm sodium acetate (NaC₂H O 3H O)
50 ml casein hydrolysate 10%
50 ml *l* cystine (4 mg per ml)
10 ml adenine guanine uracil (1 mg each per ml)
5 0 ml salt solution A
5 0 ml salt solution B
3 0 ml vitamin solution (thiamin and PABA 50 mcg per ml niacin and pyridoxine 100 mcg per ml)
3 0 ml biotin (0 1 mcg per ml)
8 0 ml riboflavin (25 mcg per ml)
3 0 ml pantothenic acid solution (50 mcg PA per ml)

b Adjust to pH 6 6 6 8 and dilute to 500 ml

4 *Preparation of Standard Tubes* Same as for riboflavin assay except for using standard tryptophane (1 mcg per ml) for the working standard

5 Preparation of assay tubes sterilization inoculation and incubation *titration* and calculation are by the same general procedures used in riboflavin assay

Precautions

1 Lactobacillus arabinosus does not respond to *d* tryptophane and complete racemization of hydrolysates prepared with *base* is assumed Therefore direct analytical results are obtained with a *dl* standard If *l* tryptophane is used as a standard the analytical results should be multiplied by 2 However recent investigations indicate that there is a good reason to believe that there is destruction of tryptophane after treatment with alkali Cystine added as a stabilizing agent affords good results

8 Agar Plate Assay for Biotin

References

1 Genghof D S Partridge C W H and Carpenter F H An Agar Plate Assay for Biotin Arch Biochem 17 413 420 (June) 1948
2 Snell E E and Wright L D A Microbiological Method for the *Determination* of Nicotinic Acid J Biol. Chem 139 675 686 (June) 1941

Principle

Biotin solutions of increasing concentrations are added to filter paper discs on a biotin free but otherwise complete medium seeded with Lactobacillus arabinosus 17 5 Graded zones of growth are produced. A linear relationship exists between the diameter of the zone of growth and the logarithm of the dose of biotin

Apparatus

1 Pen cillin reader (Fisher)
2 Petri d shes (100 mm diameter) with unglazed porcelain tops (Coors)
3 Filter paper discs (Schleicher and Schuell No 740 E)
4 Incubator set at 30 C
5 Autoclave
6 Refrigerator
7 Constant temperature bath set at 45 C
8 Pipettes (0 1 ml graduated in hundredths)
9 Centrifuge
10 Brewer automatic pipette to deliver 16 ml amounts (*Manual delivery of 16 ml is t me consuming*)
11 Graph paper semi log

Reagents

1 Water glass distilled
2 Biot n ampoules or crystalline
3 Casein hydrolysate 10%
4 *l* (−) Cystine
5 Tryptophane
6 Sodium acetate
7 Glucose
8 Potassium *monohydrogen* phosphate
9 Potassium dihydrogen phosphate
10 Sodium chloride
11 Manganese sulfate (tetrahyd ate)
12 Magnesium sulfate (heptahydrate)
13 Ferrous sulfate (heptahydrate)
14 Guanine hydrochloride } AGU (1 mg each per ml)
15 Adenine sulfate }
16 Uracil }
17 Riboflavin
18 Niac n
19 Thiamine
20 Pantothenic ac d
21 Pyridoxine
22 Para amino benzoic acid (PABA)
23 Agar
24 *Stock Culture*
The organism used is Lactobacillus arabinosus 17 5 (American Type Culture Collection Number 8014) It is maintained on yeast agar stabs.

Procedure

1 Sterilize Petri dishes at 165 C for at least 90 minutes in oven
2 Inoculate a culture of L arabinosus 17 5 into 10 ml of sterile basal med um plus 0 0002 gamma biotin per tube After incubation at 30 C for 18 0 hours the organisms are centrifuged and washed twice with 10 ml of sterile saline and resuspended in 5 ml of saline

Incubate 72 hours at 37 C with occasional mixing Peplace toluene as necessary Adjust pH to 6 0 6 5, including blank dilute to 200 ml, and filter until clear using same filter paper for repeated filtrations Make final dilution as follows blood 1 to 3000 plasma 1 to 2000 diet 1 to 400, enzyme blank 1 to 1000 on basis of pepsin urine 1 to 20

b *Use of Barium Hydroxide* Place sample containing about 250 mcg tryptophane in a 250 ml Erlenmeyer flask (diet 1 to 2 gm blood 0 1 ml plasma 0 2 ml urine, 0 2 ml) Add 6 5 gm barium hydroxide $Ba(OH)_2$ $8H_2O$) and 20 ml water, mix thoroughly and autoclave 16 hours at 15 lbs Autoclaved samples are transferred quantitatively while hot to 250 ml beakers After cooling the pH is made acid to 2 5 with 2 N H_2SO_4 Samples are neutralized to pH 6 0 6.5 with NaOH and diluted to volume plus 1 ml to allow for $BaSO_4$ formed Final dilutions are as follows food 1 to 50 or 1 to 150 depending on tryptophane content *blood 1 to 1000 plasma 1 to 500* urine 1 to 100 *Heat to 60 70 C filter and* assay *Note* Because of racemization when barium hydroxide is used multiply all assay values by 2

3 *Preparation of Basal Medium* The basal medium recommended here differs from *that of Snell and Wright s for niacin only in the omission of tryptophane* and the inclusion of niacin

a Mix together

20 gm glucose
33 4 gm sodium acetate ($NaC_2H_3O_2$ $3H_2O$)
50 ml casein hydrolysate 10%
50 ml *l* cystine (4 mg per ml)
10 ml adenine guanine uracil (1 mg each per ml)
5 0 ml salt solution A
5 0 ml salt solution B
3 0 ml vitamin solution (thiamin and PABA 50 mcg per ml niacin and pyridoxine 100 mcg per ml)
3 0 ml biotin (0 1 mcg per ml)
8 0 ml riboflavin (25 mcg per ml)
3 0 ml pantothenic acid solution (50 mcg PA per ml)

b Adjust to pH 6 6 6 8 and dilute to 500 ml

4 *Preparation of Standard Tubes* Same as for riboflavin assay except for using *standard tryptophane (1 mg per ml) for the working standard*

5 Preparation of assay tubes sterilization inoculation and incubation titration and calculation are by the same general procedures used in riboflavin assay

Precautions

1 Lactobacillus *arabinosus does not respond to d tryptophane* and complete racemization of hydrolysates prepared with *base* is assumed Therefore direct analytical results are obtained with a *dl* standard If *l* tryptophane is used as a standard the analytical results should be multiplied by 2 However recent investigations indicate that there is a good reason to believe that there is destruction of tryptophane after treatment with alkali Cystine added as a stabilizing agent affords good results

8 Agar Plate Assay for Biotin

References

1 Genghof D S Partridge C W H and Carpenter F H An Agar Plate Assay for Biotin Arch Biochem 17 413 4 0 (June) 1948

2 Snell E E and Wright L D A Microbiological Method for the Determination of Nicotinic Acid J Biol Chem 139 675 686 (June) 1941

Principle

Biotin solutions of increasing concentrations are added to filter paper discs on a biotin free but otherwise complete medium seeded with Lactobacillus arabinosus 17 5 Graded zones of growth are produced. A linear relationship exists between the diameter of the zone of growth and the logarithm of the dose of biotin

Apparatus

1 Penicillin reader (Fisher)
Petri dishes (100 mm diameter) with unglazed porcelain tops (Coors)
3 Filter paper d scs (Schleicher and Schuell No 740 E)
4 Incubator set at 30 C
5 Autoclave
6 Refrigerato
7 Constant temperature bath set at 45 C
8 Pipettes (0 1 ml graduated in hundredths)
9 Centrifuge
10 Brewer automatic pipette to deliver 16 ml amounts (Manual delivery of 16 ml is time consuming)
11 Graph paper semi log

Reagents

1 Water glass distilled.
2 Biotin ampoules or crystalline
3 Casein hydrolysate 10%
4 l (−) Cystine
5 Tryptophane
6 Sodium acetate
7 Glucose
8 Potassium monohydrogen phosphate
9 Potassium dihydrogen phosphate
10 Sodium chloride
11 Manganese sulfate (tetrahydrate)
12 Magnesium sulfate (heptahydrate)
13 Ferrous sulfate (heptahydrate)
14 Guanine hydrochloride
15 Adenine sulfate
16 Uracil
(14–16: AGU (1 mg each per ml))
17 Riboflavin
18 Niacin
19 Th amine
20 Pantothenic acid
21 Pyridoxine
22 Para amino benzoic acid (PABA)
23 Agar
24 *Stock Culture*
The organism used is Lactobacillus arabinosus 17 5 (American Type Culture Collection Number 8014) It is maintained on yeast agar stabs.

Procedure

1 *Sterilize Petri dishes at 165 C for at least 90 minutes in oven.*
Inoculate a culture of L. arabinosus 17 5 into 10 ml of sterile basal medium plus 0 000 gamma biotin per tube After incubation at 30 C for 18 0 hours the organisms are centrifuged and washed twice with 10 ml of sterile saline and resuspended in 5 ml of saline

Incubate 72 hours at 37 C with occasional mixing Peplace toluene as necessary Adjust pH to 6 0 6 5 including blank dilute to 200 ml and filter until clear, using same filter paper for repeated filtrations. Make final dilution as follows blood, 1 to 3000 plasma 1 to 2000 diet, 1 to 400, enzyme blank, 1 to 1000 on basis of pepsin urine 1 to 20

b Use of Barium Hydroxide Place sample containing about 250 mcg tryptophane in a 250 ml Erlenmeyer flask (diet, 1 to 2 gm blood 0 1 ml plasma 0.2 ml urine 0 2 ml) Add 6 5 gm barium hydroxide $Ba(OH)_2$ 8H O) and °0 ml water mix thoroughly and autoclave 16 hours at 15 lbs Autoclaved samples are transferred quantitatively while hot to 250 ml beakers After cooling the pH is made acid to 2.5 with 2 N H_2SO_4. Samples are neutralized to pH 6 0-6.5 with NaOH and diluted to volume plus 1 ml to allow for BaSO formed Final dilutions are as follows food, 1 to 50 or 1 to 100 depending on tryptophane content blood 1 to 1000 plasma 1 to 500 urine 1 to 100 Heat to 60 70 C filter and assay *Note* Because of racemization when barium hydroxide is used multiply all assay values by 2

3 *Preparation of Basal Medium* The basal medium recommended here differs from that of Snell and Wright s for niacin only in the omission of tryptophane and the inclusion of niacin

a. *Mix together*

20 gm glucose
33 4 gm sodium acetate ($NaC_2H_3O_2$ $3H_2O$)
50 ml casein hydrolysate 10%
50 ml l cystine (4 mg per ml)
10 ml adenine guanine uracil (1 mg each per ml)
5 0 ml salt solution A
5 0 ml salt solution B
3 0 ml vitamin solution (thiamin and PABA 50 mcg per ml niacin and pyridoxine 100 mcg per ml)
3 0 ml biotin (0 1 mcg per ml)
8 0 ml riboflavin (25 mcg per ml)
3 0 ml pantothenic acid solution (50 mcg PA per ml)

b Adjust to pH 6 6 6 8 and dilute to 500 ml

4 *Preparation of Standard Tubes* Same as for riboflavin assay except for using standard tryptophane (1 mcg per ml) for the working standard

5 Preparation of assay tubes sterilization inoculation and incubation, titration and calculation are by the same general procedures used in riboflavin assay

Precautions

1 Lactobacillus arabinosus does not respond to *d* tryptophane and complete racemization of hydrolysates prepared with *base* is assumed. Therefore direct analytical results are obtained with a *dl* standard If *l* tryptophane is used as a standard the analytical results should be multiplied by 2 However recent investigations indicate that there is a good reason to believe that there is destruction of tryptophane after treatment with alkali Cystine added as a stabilizing agent affords good results

8 Agar Plate Assay for Biotin

References

1 Genghof D S Partridge C W H and Carpenter F H An Agar Plate Assay for Biotin Arch. Biochem 17 413 420 (June) 1948
2 Snell E E and Wright L. D A Microbiological Method for the Determination of Nicotinic Acid J Biol. Chem. 139 675-686 (June) 1941

Principle

Biotin solutions of increasing concentrations are added to filter paper discs on a biotin free but otherwise complete medium seeded with Lactobacillus arabinosus 17 5 Graded zones of growth are produced. A linear relationship exists between the diameter of the zone of growth and the logarithm of the dose of biotin

Apparatus

1 Penicillin reader (Fisher)
Petri dishes (100 mm diameter) with unglazed porcelain tops (Coors)
3 Filter paper discs (Schleicher and Schuell No 740 E)
4 Incubator set at 30 C
5 Autoclave
6 Refrigerator
7 Constant temperature bath set at 45 C
8 Pipettes (0 1 ml graduated in hundredths)
9 Centrifuge
10 Brewer automatic pipette to deliver 16 ml amounts (Manual delivery of 16 ml is time consuming)
11 Graph paper semi log

Reagents

1 Water glass distilled.
2 Biotin ampoules or crystalline
3 Casein hydrolysate 10%
4 l (-) Cystine
5 Tryptophane
6 Sodium acetate
7 Glucose
8 Potassium monohydrogen phosphate
9 Potassium dihydrogen phosphate
10 Sodium chloride
11 Manganese sulfate (tetrahydrate)
12 Magnesium sulfate (heptahydrate)
13 Ferrous sulfate (heptahydrate)
14 Guanine hydrochloride }
15 Adenine sulfate } AGU (1 mg each per ml)
16 Uracil }
17 Riboflavin
18 Niacin
19 Thiamine
20 Pantothenic acid
21 Pyridoxine
22 Para amino benzoic acid (PABA)
23 Agar
24. *Stock Culture*
The organism used is Lactobacillus arabinosus 17 5 (American Type Culture Collection Number 8014) It is maintained on yeast agar stabs.

Procedure

1 Sterilize Petri dishes at 165 C for at least 90 minutes in oven.
Inoculate a culture of L. arabinosus 17 5 into 10 ml of sterile basal medium plus 0 000? gamma biotin per tube After incubation at 30 C for 18-20 hours the organisms are centrifuged and washed twice with 10 ml of sterile saline and resuspended in 5 ml of saline

Incubate 72 hours at 37 C with occasional mixing Replace toluene as necessary Adjust pH to 6 0 6 5 including blank dilute to 200 ml and filter until clear using same filter paper for repeated filtrations Make final dilution as follows blood 1 to 3000, plasma, 1 to 2000, diet, 1 to 400, enzyme blank 1 to 1000 on basis of pepsin urine 1 to 20

b Use of Barium Hydroxide Place sample containing about 250 mcg tryptophane in a 250 ml Erlenmeyer flask (diet 1 to 2 gm, blood 0 1 ml plasma 0.2 ml urine 0 2 ml) Add 6 5 gm barium hydroxide, $Ba(OH)_2 8H_2O$) and 20 ml water mix thoroughly and autoclave 10 hours at 15 lbs Autoclaved samples are transferred quantitatively while hot to 250 ml beakers After cooling the pH is made acid to 2 5 with 2 N H_2SO_4 Samples are neutralized to pH 6 0 6 5 with NaOH and diluted to volume plus 1 ml to allow for $BaSO_4$ formed Final dilutions are as follows food 1 to 50 or 1 to 150 depending on tryptophane content blood 1 to 1000 plasma 1 to 500 urine 1 to 100 Heat to 60 70 C filter and assay *Note* Because of racemization when barium hydroxide is used multiply all assay values by 2

3 *Preparation of Basal Medium* The basal medium recommended here differs from that of Snell and Wright's for niacin only in the omission of tryptophane and the inclusion of niacin

a. Mix together

20 gm glucose
33 4 gm sodium acetate ($NaC_2H_3O_2 3H_2O$)
50 ml casein hydrolysate 10%
50 ml *l* cystine (4 mg per ml)
10 ml adenine guanine uracil (1 mg each per ml)
5 0 ml salt solution A
5 0 ml salt solution B
3 0 ml vitamin solution (thiamin and PABA 50 mcg per ml niacin and pyridoxine 100 mcg per ml)
3 0 ml biotin (0 1 mcg per ml)
8 0 ml riboflavin (25 mcg per ml)
3 0 ml pantothenic acid solution (50 mcg PA per ml)

b Adjust to pH 6 6 6 8 and dilute to 500 ml

4 *Preparation of Standard Tubes* Same as for riboflavin assay except for using standard tryptophane (1 mcg per ml) for the working standard

5 Preparation of assay tubes sterilization inoculation and incubation titration and calculation are by the same general procedures used in riboflavin assay

Precautions

1 Lactobacillus arabinosus does not respond to *d* tryptophane and complete racemization of hydrolysates prepared with *base* is assumed Therefore direct analytical results are obtained with a *dl* standard If *l* tryptophane is used as a standard the analytical results should be multiplied by 2 However, recent investigations indicate that there is a good reason to believe that there is destruction of tryptophane after treatment with alkali Cystine added as a stabilizing agent affords good results

8 Agar Plate Assay for Biotin

References

1 Genghof D S Partridge C W H and Carpenter F H An Agar Plate Assay for Biotin Arch Biochem 17 413 4 0 (June) 1948
2 Snell, E E and Wright L D A Microbiological Method for the Determination of Nicotinic Acid J Biol Chem 139 675 686 (June) 1941

Principle

Biotin solutions of increasing concentrations are added to filter paper discs on a biotin free but otherwise complete medium seeded with Lactobacillus arabinosus 17 5 Graded zones of growth are produced. A linear relationship exists between the diameter of the zone of growth and the logarithm of the dose of biotin

Apparatus

1 Penicillin reader (Fisher)
2 Petri dishes (100 mm diameter) with unglazed porcelain tops (Coors)
3 Filter paper discs (Schleicher and Schuell No 740 E)
4 Incubator set at 30 C
5 Autoclave
6 Refrigerator
7 Constant temperature bath set at 45 C
8 Pipettes (0 1 ml graduated in hundredths)
9 Centrifuge
10 Brewer automatic pipette to deliver 16 ml amounts (Manual delivery of 16 ml is time consuming)
11 Graph paper semi log

Reagents

1 Water glass distilled
2 Biotin ampoules or crystalline
3 Casein hydrolysate 10%
4 l (−) Cystine
5 Tryptophane
6 Sodium acetate
7 Glucose
8 Potassium monohydrogen phosphate
9 Potassium dihydrogen phosphate
10 Sodium chloride
11 Manganese sulfate (tetrahydrate)
12 Magnesium sulfate (heptahydrate)
13 Ferrous sulfate (heptahydrate)
14 Guanine hydrochloride } AGU (1 mg each per ml)
15 Adenine sulfate }
16 Uracil }
17 Riboflavin
18 Niacin
19 Thiamine
20 Pantothenic acid
21 Pyridoxine
22 Para amino benzoic acid (PABA)
23 Agar
24 *Stock Culture*
The organism used is Lactobacillus arabinosus 17 5 (American Type Culture Collection Number 8014) It is maintained on yeast agar stabs.

Procedure

1 Sterilize Petri dishes at 165 C for at least 90 minutes in oven
2 Inoculate a culture of L. arabinosus 17 5 into 10 ml of sterile basal medium plus 0 0002 gamma biotin per tube After incubation at 30 C for 18-20 hours the organisms are centrifuged and washed twice with 10 ml of sterile saline and resuspended in 5 ml of saline

Incubate 72 hours at 37 C with occasional mixing Replace toluene as necessary Adjust pH to 6 0 6 5, including blank dilute to 200 ml and filter until clear, using same filter paper for repeated filtrations Make final dilution as follows blood, *1 to 3000 plasma 1 to 2000 diet 1 to 400*, enzyme blank, 1 to 1000 on basis of pepsin urine 1 to 20

b Use of Barium Hydroxide Place sample containing about 250 mcg tryptophane in a 250 ml Erlenmeyer flask (diet 1 to 2 gm blood, 0 1 ml plasma 0 2 ml urine 0 2 ml) Add 6 5 gm barium hydroxide $Ba(OH)_2 \cdot 8H_2O$) and 20 ml water mix thoroughly and autoclave 16 hours at 15 lbs Autoclaved samples are transferred quantitatively while hot to 250 ml beakers After cooling the pH is made acid to 2 5 with 2 N H_2SO_4 Samples are neutralized to pH 6 0 6 5 with NaOH and diluted to volume plus 1 ml to allow for $BaSO_4$ formed Final dilutions are as follows food 1 to 50 or 1 to 150 depending on tryptophane content blood 1 to 1000 plasma 1 to 500, urine 1 to 100 Heat to 60 70 C filter and assay *Note* Because of racemization when barium hydroxide is used multiply all assay values by 2

3 *Preparation of Basal Medium* The basal medium recommended here differs from that of Snell and Wright s for niacin only in the omission of tryptophane and the inclusion of niacin

a Mix together

20 gm glucose
33 4 gm sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$)
50 ml casein hydrolysate 10%
50 ml *l* cystine (4 mg per ml)
10 ml adenine guanine uracil (1 mg each per ml)
5 0 ml salt solution A
5 0 ml salt solution B
3 0 ml vitamin solution (thiamin and PABA 50 mcg per ml niacin and pyridoxine 100 mcg per ml)
3 0 ml biotin (0 1 mcg per ml)
8 0 ml riboflavin (25 mcg per ml)
3 0 ml pantothenic acid solution (50 mcg PA per ml)

b Adjust to pH 6 6 6 8 and dilute to 500 ml

4 *Preparation of Standard Tubes* Same as for riboflavin as ay except for using standard tryptophane (1 mcg per ml) for the working standard

5 Preparation of assay tubes sterilization inoculation and incubation titration and calculation are by the same general procedures used in riboflavin assay

Precautions

1 Lactobacillus arabinosus does not respond to *d* tryptophane and *complete* racemization of hydrolysates prepared with *base* is assumed Therefore direct analytical results are obtained with a *dl* standard If *l* tryptophane is used as a standard the analytical results should be multiplied by 2 However recent investigations indicate that there is a good reason to believe that there is destruction of tryptophane after treatment with alkali Cystine added as a stabilizing agent affords good results

8 Agar Plate Assay for Biotin

References

1 Genghof D S Partridge C W H and Carpenter F H An Agar Plate Assay for Biotin Arch Biochem 17 413 420 (June) 1948
2 Snell E E and Wright L D A Microbiological Method for the Determination of Nicotinic Acid *J Biol Chem 139 675 686 (June) 1941*

3 Prepare the following medium

Casein hydrolysate 10%	50 ml
l (−) Tryptophane	100 mg
l (−) Cystine	100 mg
Glucose	10 gm
$NaC_2H_3O_2$	6 gm
AGU	10 mg
Salt A	5 ml
Salt B	5 ml
Niacin	500 gamma
PABA	100 gamma
Calcium pantothenate	100 gamma
Pyridoxine hydrochloride	150 gamma
Thiamine	100 gamma
Riboflavin	[illegible]00 gamma
Bacto agar	15 gm

Adjust to pH 6 6 6 8 and dilute up to 1 liter

4 Autoclave the medium for 15 minutes at 120 C cool to 45 C and inoculate with resuspended cells (20 ml of the Lactobacillus arabinosus suspension per liter of medium)

5 To each sterile Petri dish add 16 ml of the above inoculated medium Place plates in refrigerator as soon as the agar is hardened These plates can be stored at 5 C for at least one week before use

6 *Standard Curve* Range 1 milligamma to 1000 milligamma of biotin Biotin solutions of the following concentration are made

1 0 milligamma per ml
2 5 milligamma per ml
5 0 milligamma per ml
10 0 milligamma per ml
25 0 milligamma per ml
50 0 milligamma per ml
100 0 milligamma per ml
250 0 milligamma per ml
500 0 milligamma per ml
1000 0 milligamma per ml

7 Filter paper discs are placed on the agar and 0 08 ml of the biotin solutions delivered upon them Run all dilutions in duplicate with 2 filter pads per plate through 10 milligamma for other dilutions only 1 pad per plate

8 Invert plates and incubate for 18 20 hours at 30 C

9 After incubation the diameters of growth about the filter pads are measured to the closest 0.5 mm with the penicillin reader

10 A standard curve is plotted on semi log graph paper The mean diameter of the circle of growth is the abcissa the log of the concentration of biotin is the ordinate A linear relationship exists between the diameter of the growth zone in mm and the log of the dose

11 After urine samples are adjusted to pH 6 6 6 8 with 2 N HCl or 4 N NaOH and the volume change noted if any the samples are carried through the same procedure as the standard solutions of biotin

Calculation

1 From the standard curve read directly the concentration of biotin in milligamma per ml It is preferable to have no dilution of urine samples If there is a volume change due to adjustment of pH multiply the direct reading by the dilution factor

Example

1 On the standard curve 25 milligamma of biotin per ml gave a reading of 27 mm on the Penicillin reader A specimen of urine, undiluted produced a growth zone of 27 mm. Thus the sample had 25 0 milligamma biotin per ml. If the sample had been diluted 9 ml of urine plus 1 ml of acid or base when adjusting pH and the zone of growth read 27 mm the sample tested would contain

$$25\,0 \times \frac{10}{9} = 27.8 \text{ milligamma/ml}$$

Precautions

1 Since the filter pads rapidly absorb water from the moist agar the solutions must be added to the pads as soon as possible after they have been placed upon the agar
2 The same volume of fluid must be delivered to each pad. Care is required at this step in the procedure
3 After inoculation of agar with organisms do not flame the flask each time before or after adding the 16 ml amounts to Petri dishes
4 Agar for inoculation must not be over 45 C nor cool enough that the agar starts to harden

9 Microbiology Miscellaneous References

Microbiology—General

Snell E E Microbiological Methods in Vitamin Research Look Vitamin Methods by Paul Gyorgy Academic Press Inc New York pages 3 305 1950

Amino Acids

Dunn M. S Determination of Amino Acids by Microbiological Assay Physiol. Rev 29 219 259 (July) 1949

Guirard B M Snell, E E., and Williams R J Microbiological Determination of Amino Acids IV Lysine, Histidine Arginine and Valine Proc Soc Exper Biol. & Med. 61 (?) 158 161 (Feb.) 1946

Schweigert B S and Snell E E Microbiological Methods for the Estimation of Amino Acids Nutrition Abst & Rev 16 (3) 497 510 (Jan) 194

Woodson H W Hier S W, Solomon J D and Bergeim, O Urinary Excretion of Amino Acids by Human Subjects on Normal Diets J Biol Chem. 172 (?) 613 618 (Feb) 1948

Arginine

Horn M J Jones D B and Blum A E Microbiological Determination of Arginine in Proteins and Foods J Biol. Chem. 176 59 63 (Oct.) 1948

Choline

Dandelin F J The Microbiological Determination of Choline J Am Pharm. A. (Scient. Ed.) 38 (6) 304 307 (June) 1949

Horowitz N H., and Beadle G W A Microbiological Method for the Determination of Choline by Use of a Mutant of Neurospora J Biol Chem 150 (2) 325-333 (Oct) 1943

Luecke R. W and Pearson P B The Microbiological Determination of Free Choline in Plasma and Urine J Biol. Chem. 153 (1) 59 63 (April) 1944

Glutamic Acid

Dunn M S Camien M N, Shankman S and Block H The Determination of d (−) and l (+)-Glutamic Acid in Protein Hydrolysates, J Biol. Chem. 168 (1) 43-49 (April) 1947

Histamine

Chen, G, Ansor, C R and Clarke I G The Biological Assay of Histamine and Diphenhydramine Hydrochloride (Benadryl Hydrochloride) J Pharmacol. and Exp. Therapeutics 97 90 9 (Jan.) 1948

Isoleucine

Horn M J., Jones, D B. and Blum, A. E. Microbiological Determination of Isoleucine in Proteins and Food J Biol. Chem. 180 695 01 (Sept) 1949

Lysine

Horn M. J Jones D B, and Blum A. E Microbiological Determination of Lysine in Proteins and Foods, J Biol. Chem 169 (1) 71 76 (June) 1947

Manganese

Bentley O G, Snell E E. and Phillips P H A Microbiological Method for the Determination of Manganese J Biol Chem 170 (1) 343 350 (Sept) 1947

Pantothenic Acid

Buskirk H. H Bergdahl, A M, and Delor P A Enzymatic Digestion of Samples for Microbiological Assay of Pantothenic Acid J Biol Chem 172 (2) 671 675 (Feb) 1948

Para-Amino Benzoic Acid

Landy M and Dicken D M A Microbiological Method for the Determination of p Amino Benzoic Acid, J Biol. Chem 146 109 114 (Nov) 1942
Lewis J C. A Lactobacillus Assay Method for p Amino Benzoic Acid, J Biol. Chem 146 441 450 (Dec) 1942
Thompson R C Isbell E P and Mitchell H K A Microbiological Assay Method for p-Amino Benzoic Acid J Biol Chem 148 281 287 (May) 1943

Phenylalanine

Prescott B A Borek, E Brecher A. and Waelsch H Studies on Oligophrenia Phenylpyruvica I. Microbiological Determination of L- and D-Phenylalanine and of Phenyllactic Acid J Biol Chem 181 273 280 (Nov) 1949

Progesterone

Hooker, C W and Forbes T R. A Bio Assay for Minute Amounts of Progesterone, Endocrinol 41 158 169 (Aug) 1947

Pteroic Acid

Franklin, A L. Stokstad E L R and Jukes T H. Urinary Excretion Studies Following the Administration of Pteroic Acid Proc Soc Exper Biol & Med 66 (3) 576-5 8 (Dec) 1947

Riboflavin

Roberts E C and Snell E E An Improved Medium for Microbiological Assays With Lactobacillus Casei J Bio Chem 163 (2) 499 509 (May) 1946

Sterols

Krámli A and Horvath, J Microbiological Oxidation of Sterols, Nature 163 19 (Feb) 1949

Tryptophane

Dunn M S, Schott H F., Frankl W and Rockland L B Investigation of Amino Acids Peptides and Proteins XX The Determination of Apparent Free Tryptophane in Blood by a Microbiological Method J Biol Chem 157 387 394 (Jan) 1945

Vitamin B_{12}

Capps B F Hobbs N L, and Fox S H A Method for the Microbiological Assay of Vitamin B_{12}, J Biol Chem 178 517 518 (March) 1949
Caswell M C Koditschek L K and Hendlin D The Microbiological Estimation of Lactobacillus Lactis Dorner Activity With Vitamin B_{12} as a Standard J Biol. Chem 180 125 132 (Aug) 1949
Hoffmann, C E, Stokstad E L R Hutchings B L, Dornbush, A C and Jukes T H The Microbiological Assay of Vitamin B_{12} With Lactobacillus Leichmannii J Biol Chem 181 635 644 (Dec) 1949
Rickes, E L, Brink N G, Koniuszy F R, Wood T R Folkers, K Vitamin B_{12} A Cobalt Complex Science 108 134 (Aug) 1948
Shorb M S Activity of Vitamin B_{12} for the Growth of Lactobacillus Lactis Science 107 397 398 (April) 1948

SECTION VI

PHYSIOLOGICAL MEASUREMENTS

A BLOOD GASES

1 Calculation of Gas Volumes

For many physiological purposes it becomes necessary to compute gas volumes dry at 0°C and 760 mm Hg barometric pressure from observations made on gas saturated with water vapor at any temperature and barometric pressure

The general equation for this calculation is

$$\text{ml gas dry at 0 C and 760 mm Hg} = \text{ml observed} \times \frac{(273.2)}{T + 273.2} \times \frac{(B - V)}{(760)}$$

Where T is ambient temperature in °C B is ambient pressure in mm Hg and V is water vapor tension at the ambient temperature a value which must be obtained from empirical tables in standard handbooks and 273 2 is the absolute temperature for 0°C

The line chart (Fig 25) was prepared by Dr Robert C Darling it gives at once the factor by which observed gas volume is multiplied to reduce it to volumes dry at 0°C and 760 mm Hg

2 Techniques for Obtaining Arterial Blood and for Handling Blood Anaerobically

Usual Method for Obtaining Arterial Blood

Arterial blood is obtained by a procedure quite similar to that for venous blood. A # 0 gauge needle 1½ inches long with a short concave bevel is preferable Particular attention is given to oiling the syringe Care should be taken that the oil is free from air bubbles and air bubbles should not be introduced during the aspiration of blood into the syringe Under no circumstances is a tourniquet to be applied The brachial artery just proximal to the antecubital fossa is usually selected for puncture It may be helpful to hyperextend the arm slightly during an arterial puncture by inserting a small pad under the elbow The position of the artery is palpated with the left index finger Arterial punctures are performed mainly by palpation rather than by observation of the vessel Attention should be given to avoid penetrating a superficial vein before entering the artery The skin is cleansed with alcohol The syringe is held in the right hand at an angle of 45 and the skin and subcutaneous tissue entered The needle and syringe are continued along a plane parallel to the artery As soon as the artery is entered blood should flow into the syringe so that little or no decompression of the plunger is needed. Once the needle is in the artery its position should not be altered until the desired quantity of blood is removed. A large sponge is then applied forcefully over the assumed site of entrance into the artery and the syringe is removed A firm bandage is immediately applied over the sponge with the pressure on and above the puncture never below

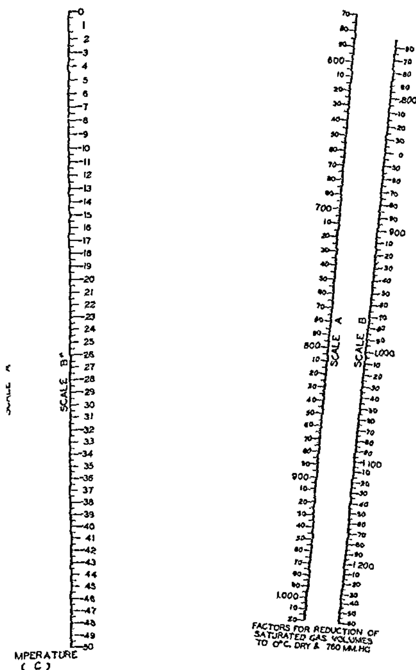


Fig 25—Line chart for converting wet gas volumes to STP and dry (This chart was prepared by Dr Robert C Darling)

The blood is next transferred to a blood tube which contains an anticoagulant and ½ inch layer of mineral oil During the initial stages of the transfer the syringe is held in a horizontal position and the plunger is not touched The needle is removed and replaced by a rubber connection to a 6 inch capillary glass tube The capillary tube is filled with blood by gentle compression of the plunger The tip of the tube is next placed at the bottom of the blood tube below the oil and the remainder of the blood is gently delivered into the bottom of the tube The blood may be mixed with the anticoagulant by repeated exchange of blood between syringe and tube meanwhile avoiding all air bubbles An alternate procedure is gently stirring the blood in the tube with a glass stirring rod taking care to avoid emulsifying the blood with the oil

Technique of Goldschmidt and Light for Arterializing Venous Blood (J Biol. Chem 64 53 1925)

The hand is immersed in hot water at 45 C for 20 minutes Blood is drawn anaerobically from any convenient vein on the back of the hand

Technique of Lilienthal and Riley for Arterializing Venous Blood (J Clin. Investigation 23 904 1944)

The ear is heated by radiant heat for 10 minutes at an air temperature of 45 C measured next to the pinna. The lobe is pricked and drops of blood are caught in oxalate or heparin in a specially constructed funnel from which samples may be drawn with a micropipette

Mook s Technique for Handling Blood Anaerobically (Biochem Z 242 3 9 1931)

One uses small glass tubes of length about 35 mm internal diameter about 3 mm and total content about 50 cmm Each tube has a slight flare to the bottom

The warmed finger is pricked and a tube is firmly applied to the hole The diffusion of CO_2 out of the blood and O into it under these conditions is such that when the tube is filled samples may be drawn with micropipettes from the center of the tube and the results are essentially the same as if the samples were drawn under oil

Scholander s Technique for Handling Blood Anaerobically (J Biol Chem 148 541 1943 See Figs 6 and 7)

The plunger of an all glass 2 or 5 ml syringe is lubricated with 1 or 2 drops of heavy oil A little heparin solution is drawn into the syringe and the walls of the barrel moistened with the solution by drawing the plunger all the way down and pushing it up again the dead space at the top and the nozzle being left full of heparin. The solution is freed from air bubbles by rapid twisting of the syringe in the vertical position the syringe being held by the nozzle or needle the plunger being arrested by a clip This procedure centrifuges the bubbles to the center so that they can be easily expelled The nozzle is attached to a needle and the blood vessel punctured The air bubble from the needle is cautiously expelled the needle removed and the nozzle dipped into a dish containing mercury a little of which is drawn into the syring The air bubble from the needle can be avoided by inserting the needle into the blood vessel with another syringe drawing the needle full of blood and then exchanging the first syringe for the syringe containing heparin A ¼ inch length of rubber tube is joined to the nozzle filled with blood and closed with a glass plug without trapping an air bubble The mercury is used for mixing the blood in the syringe which is immersed in a beaker of ice and water For high accuracy it may be necessary to correct for the slight dilution with the heparin solution.

For transfer to the pipette the syring is removed from the beaker dried the blood well mixed and the fluid in the rubber tube pumped a few times into the main bulk of the blood by working the glass plug up and down The plug and rubber tube are then removed and the pipette with the tip protruding about an inch over the edge of the bench is held with its conical tip pressed against the opening of the syringe nozzle. If difficulty

and

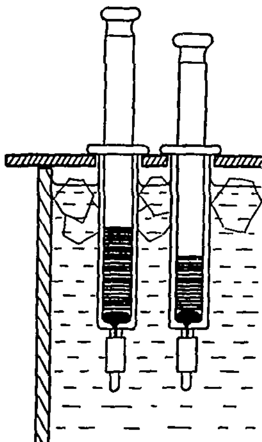


Fig 26 —Anaerobic storage of blood by Scholander's technique

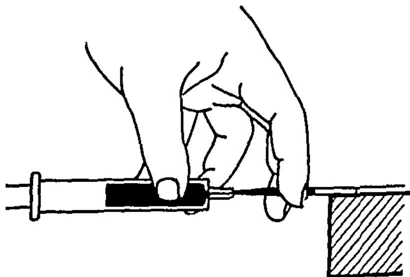


Fig 27 —Transfer of blood from storage syringe into pipette.

is experienced in this step the pipette tip may be covered with a rubber plug with a capillary bore The pipette is then loaded by screwing in the plunger The syringe is restoppered without trapping air and returned to the ice bath

3 The Van Slyke-Neill Manometric Apparatus

Reference

Van Slyke D D and Neill J M The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurements J Biol Chem 61 5 3 538 (Sept) 19 4

Principle

In the Van Slyke apparatus advantage is taken of a vacuum The gas is liberated chemically extracted by vacuum and its partial pressure measured in a partial vacuum on a mercury manometer calibrated in centimeters All reagents employed must be free from physically dissolved gases in significant amounts

Apparatus

(See Fig 28)

Preparation of Air Free Solutions

Dissolved gases are removed from the solution by adding a suitable volume of reagent to the chamber sealing the stopcock to the chamber with mercury and shaking the evacuated chamber This is done by lowering the leveling bulb and opening the leveling stopcock The liquid is allowed to fall to the point where the *mercury is approximately at the 50 ml mark in the chamber Shaking is allowed to* proceed for three minutes then the machine is stopped and the mercury stopcock is opened allowing the solution to come to atmospheric pressure The leveling bulb is now raised to the higher bracket on the machine and the stopcock to the chamber is opened allowing only the gas to escape Repeat the procedure again

About one ml of mercury is now added to the cup and a storing device usually some form of separatory funnel containing mineral oil or a burette seated in the chamber cup The mercury acts as a gasket. The stopcock to the storage apparatus is opened and this is followed by opening the chamber stopcock forcing the fluid up into the storage tower where it is stored under oil

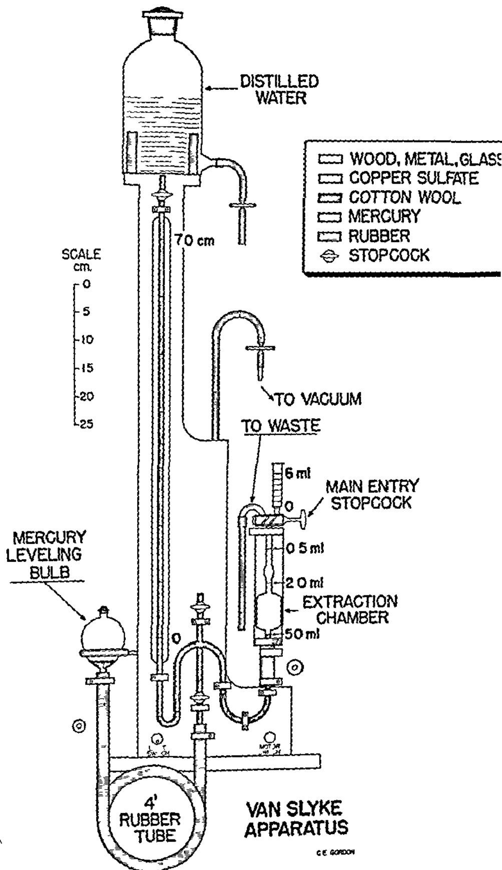
Most reagents are evacuated and stored in this manner Under the heading *Reagents* in each method the reagents that need evacuating and storage in this manner will be mentioned.

Machine Correction (C Corrections)

In all gasometric determinations a machine correction must be established (C) This is done by using the same procedure as for blood except that where blood is used a substituted volume of air free water is used or in the case of the determination of oxygen 1 ml extra oxygen reagent is substituted for blood. The C correction is really a correction that compensates for the pressure change due to the *addition of the absorption reagents For oxygen the C correction is about 10* mm for carbon dioxide about 15 0 mm of mercury

Moisture in the Manometer

Small amounts of moisture find their way into the manometer as the mercury flows back and forth from the analysis chamber Some error from variation of the vapor pressure might occur if it were left in the manometer tube To absorb the water vapor a few drops of ethylene glycol (E. K. Co.) are admitted through the stopcock at the top of the manometer and permitted to flow down the tube for 10 cm. Allow the fluid to run up and down the manometer by raising and lowering the mercury leveling bulb Finally the fluid is forced through the top stopcock and fluid is left behind but not enough to interfere with the readings of the mercury meniscus. Renew occasionally in the same manner



DISTILLED WATER
WOOD, METAL, GLASS
COPPER SULFATE
COTTON WOOL
MERCURY
RUBBER
STOPCOCK
70 cm
SCALE cm.
0
5
10
15
20
25
TO VACUUM
TO WASTE
6 ml
0
MAIN ENTRY STOPCOCK
0.5 ml
2.0 ml
EXTRACTION CHAMBER
50 ml
MERCURY LEVELING BULB
0
4' RUBBER TUBE
VAN SLYKE APPARATUS
C.E. GORDON

Fig 25

Precautions

1 Always remember two things
   a. Whenever taking in samples or solutions the leveling bulb *must* always be on the lower support
   b Whenever expelling any mixture from the chamber always place the leveling bulb in the upper support
2 When the chamber has been evacuated *never* open the main stopcock as the vacuum will send the mixture in the chamber back into the manometer

1 Oxygen in Whole Blood (Van Slyke)

Reference

Van Slyke D D and Neill J M The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurements VII. The Determination of Oxygen in Blood J Biol Chem 61 55 554 (Sept) 19 4

Principle

Oxygen in blood is released from oxyhemoglobin by shaking in a vacuum with potassium ferric anide The liberated oxygen is measured manometrically by absorbing the oxygen with sodium hydrosulfite

Apparatus

1 A Van Slyke manometric apparatus with an electric motor
  Van Slyke Neill stopcock pipettes calibrated between marks capacities of 0.5 ml 1 0 ml and 0 ml
3 Two 50 ml burettes for storing air free sodium hydroxide (NaOH) and sodium hydrosulfite (Na_2S_2O) solutions Keep two to three inches of oil in the burettes to prevent the solution from coming into contact with the outside air
4 Rubber tips for delivering sample into evacuating chamber

Reagents

1 Oxygen reagent 3 gm of pure white saponin 6 gm of potassium ferricyanide and 3 ml caprylic alcohol Dissolve and dilute to 1000 ml with water Shake well before using
2 Sodium hydroxide Approximately 1 N Dissolve 40 gm NaOH in water and dilute to 1000 ml. Make about 5 30 ml air free by a shaking in a vacuum two to three times and expel gas between shakings Store in 50 ml burette under oil Make up air free NaOH each day
3 Sodium hydrosulfite ($Na_2S_2O_4$, Lycopon) Mix 5 gm quickly in 0 5 ml of 1 N NaOH and make air free as soon as possible Store in the other 50 ml burette under paraffin oil. Do not try to dissolve any more than will go into solution by merely rotating flask for a few seconds Make fresh daily
4 Fieser s modified reagent (may be used for absorbing the oxygen) Dissolve in the following order in 100 ml of distilled water 14 gm of potassium hydroxide 16 gm of sodium hydrosulfite 3 gm sodium anthraquinone beta sulfonate and add 3 drops of 10% ferric chloride solution Store in rubber toppered flask in the cold Make fresh every week
5 Mercury Although a cleaning tower may be used a simpler device is to use an aspirating bottle By bubbling air through the mercury in the latter case the mercury is brought into contact with the dilute HNO_3. Bubble air through the Hg for several hours Remove HNO_3 and repeat with several washes using distilled water Repeat once with glass-distilled water Drain off mercury and absorb visible water with filter paper
6 Stopcock grease (Lubriseal A. H Thomas Co)

7 Lactic acid solution approximately 1 N Use C P lactic acid solution and dilute 92 ml to 1000 ml with distilled water
8 Caprylic alcohol (Eastman Kodak Co)
9 Oil (heavy paraffin)

Procedure

1 Rinse out apparatus by shaking 2 ml of 1 N lactic acid solution plus 10 ml of distilled water in a vacuum Expel into waste jar
2 The waste bore of the chamber stopcock is sealed with mercury Vacuum chamber and main bore of main cock are also filled with mercury Main cock is closed Leveling cock is open Mercury bulb is at atmospheric level (lower level)
3 Pour 6 ml oxygen reagent and 2 drops of caprylic alcohol into entry cup *Open main cock and take in reagent to top of capillary between cup and chamber* Close cock
4 Seal chamber and cock Pour a few drops of mercury into cup Open and close cock so that main bore fills with mercury from end to end. Suck off excess from cup
5 Evacuate solution Drop mercury level to 50 ml mark in chamber (Open leveling cock and lower bulb until sufficient mercury has left chamber Then close cock.) Shake gently for 3 minutes
6 Expel air from chamber Raise bulb to high level. Open leveling cock slowly (so that mercury will not strike top of manometer tube suddenly) Open main cock slowly and release air Let out reagent to 5 5 ml mark in cup Close main cock and support bulb at atmospheric level.
7 Admit blood Using main cock take in exactly 1 ml blood from a Van Slyke pipette and take oxygen reagent into cup to the 3 5 ml mark Close cock and seal Suck off excess fluid and mercury
8 Evacuate Drop mercury level to 50 ml mark as above Shake for 3 minutes
9 Absorb carbon dioxide Reduce gas phase to about 5 ml by opening and closing leveling cock Add 1 ml NaOH (air free) to cup Take in 0 5 ml drop by drop by manipulation of main cock Seal and suck off mercury and remains of the reagent *Let reagent drain for a minute*
10 Read P Adjust gas phase to 2 ml mark at a constant rate using leveling cock. P is the pressure of gaseous oxygen and nitrogen Read manometer to the nearest 1/10 mm.
11 *Absorb oxygen Add 1 ml hydrosulfite solution to cup and take in 0 5 ml drop* by drop over a period of 2 minutes When half has been added open leveling cock part way and allow contents to rise in chamber while remainder is added. Seal and suck off excess fluid cup There should be only a tiny bubble of nitrogen at top of vacuum chamber when oxygen is completely absorbed.
12 Read P Adjust gas phase to 2 ml mark using leveling cock and read manometer to nearest 1/10 mm
13 Record the temperature of the chamber to the nearest 1/10 C
14. Release pressure and expel the mixture into the waste jar
15 Rinse out with lactic acid and water again and expel

Calculation

1 mm partial pressure *total O at T of water jacket* $= P_1 - P - C$
2 ml total O_2/100 ml blood =
(mm partial pressure O) × (temperature factor from Table 16)
3 ml O_2 bound by hemoglobin/100 ml blood =
(ml total O − ml dissolved O at T and pO observed)
a For venous blood dissolved O is 0 10 ml/100 ml
b For arterial blood dissolved O is 0 ?5 ml/100 ml.

TABLE 16

OXYGEN FACTORS (VAN SLYKE)

To obtain ml O_2/100 ml blood multiply pO at jacket temperature by factor in table (One ml samples analyzed)

JACKET TEMP C	O_2 FACTOR	JACKET TEMP C	O FACTOR	JACKET TEMP C	O FACTOR	JACKET TEMP C	O FACTOR
1ɔ 0	..493	0 0	..450	ɔ 0	406	ɔ0 0	ɔ66
1	9	1	49	1	0ɔ	1	6ɔ
˷	91	°	48	°	04	2	64
3	91	3	47	3	03	3	64
4	90	4	46	4	03	4	63
.5	89	.5	45	›	0	5	6
6	88	6	4›	ϵ	01	6	61
7	87	7	44		°400		60
8	87	8	43	8	°400	8	60
9	86	9	4	9	399	9	9
16 0	485	1 0	..441	6 0	..398	31 0	..ɔɔ8
1	84	1	40	1	97	1	57
	84	°	39	.2	96	2	56
3	83	3	38	3	96	3	56
4	8	4	37	4	9	4	5ɔ
5	81	.5	36		94	5	ɔ4
6	81	6	36	6	93	6	53
7	80	7	3	7	9	7	
8	79	8	34	8	9	8	
9	7ɔ	9	33	9	91	9	ɔ1
1 0	4 8	0	4	0	390	ɔ 0	3ɔ0
1	77	1	31	1	89	1	49
	7f	°	0	°	88	.2	48
3	ɔ	3	9	3	88	3	48
4	4	4	°8	4	8	4	47
ɔ	73	ɔ		5	86	5	46
6	7	6	7	6	8ɔ	6	4
7	71	7	°6		84	7	44
8	70	8	ɔ	8	81	8	44
9	69	9	°4	9	83	9	43
18 0	468	3 0	4 1	8 0	..383	33 0	..34
1	67	1		1	81	1	41
	66	°	°1	°	80	.2	40
3	66	3	0	3	9	.3	39
4	6›	4	19	4	9	4	38
ɔ	64	5	18	5	8	.5	3
6	63	6	18	6	7	6	3
	6		1	7	6	·	36
8	61	8	16	8	6	8	35
9	60	9	1ɔ	9	ɔ	9	34
11 0	45ɔ	4 0	414	9 0	..3 4	34 0	333
1	58	1	13	1	73		
	57	°	1°	.2			
3	56	3	1°	.3	-		
4	55	4	11	4	1		
5	54	.5	10	5	0		
6	51	6	09	6	67		
	53	7	08	7	68		
8	5	8	08	8	68		
9	51	9	0	9	6		

c For blood equilibrated with air at 760 mm and 25 C dissolved O is 0 6 ml/100 ml

4 gm hemoglobin/100 ml blood = $\frac{\text{(ml bound oxygen)}}{1\ 34}$

Example

P = 153 2 mm P = 72 5 mm C = 0 7 mm T = 25 C

1 *ml total O_2/100 ml = (153 2 - 72 5 - 0 7) × (0 2406) = 19 25*

2 ml dissolved O /100 ml = 0 62

3 ml bound O = 18 63/100 ml blood

4 gm Hb/100 ml blood = 18 63/1 34 = 13 9

Precautions

See section on apparatus

5 Carbon Dioxide in Whole Blood and Serum (Van Slyke)

Reference

Van Slyke D D and Neill J M The Determination of Gases in Blood by Vacuum Extraction and Manometric Measurements V *Determination of Carbon Dioxide* J Biol Chem 61 543 553 (Sept) 1924

Principle

Both bound carbonate and free carbon dioxide are liberated from the blood or serum with lactic acid and the liberated carbon dioxide is measured manometrically by absorption with sodium hydroxide

Apparatus

The same as for Oxygen.

Reagents

1 Caprylic alcohol (Eastman Kodak Co)
2 Distilled water Make air free and store under oil as for oxygen reagents.
3 Sodium hydroxide approximately 1 N 40 gm dissolved in 1000 ml distilled water Make air free
4 Lactic acid approximately 1 N 92 ml USP lactic acid diluted to 1000 ml with water
5 Very clean mercury (See oxygen method)
6 Paraffin oil heavy

Procedure

1 *Clean out apparatus by shaking in a vacuum with 2 ml lactic acid solution* and approximately 10 ml of distilled water Shake approximately one minute and expel into waste jar
2 Seal off the main stopcock at both ends with mercury by raising the leveling bulb to the upper position
3 *Place leveling bulb at lower position and admit one to 2 drops of caprylic alcohol* into the capillary of the main stopcock
4 Place 2 3 ml of air free water into the entry cup
5 Pipette exactly 1 ml of serum or blood (when using blood make certain that the blood specimen has been stirred well as too much of either the red cells or plasma will cause *very erroneous results) and introduce into the chamber through a rub* ber tip
6 Then wash in the sample with the remainder of the water
7 Add 6 drops (0 3 ml) of lactic acid solution and admit into the chamber
8 Seal the main stopcock with mercury and draw off the excess mercury in the cup by means of a vacuum

TABLE 17

CO FACTORS FOR VAN SLYKE APPARATUS

(For 1 ml samples of blood or serum To obta n ml total CO per 100 ml sample multiply pCO by factor for jacket temperature)

JACKET TEMP C	CO FACTOR	JACKET TEMP C	CO FACTOR	JACKET TEMP C	CO_2 FACTOR	JACKET TEMP C	CO FACTOR
15 0	735	0 0	66	5 0	594	30 0	533
1	3	1	61	1	93	1	[illegible]
	30		60		91	2	31
3	8	3	59	3	90	3	30
4	6	4	57	4	89	4	[illegible]
	4	5	5	5	88	5	
6	23	6	54	6	86	6	6
7	2	7	53	7	[illegible]		5
8	21	8	52	8	84	8	4
9	0	9	50	9	83	9	3
16 0	719	21 0	648	0 0	581	31 0	5
1	17	1	47	1	80	1	1
	16		46		79		0
3	15	3	45	3	77	3	19
4	13	4	44	4	76	4	18
5	11	5	43	5	5	5	16
6	10	6	41	6	74	6	15
7	8	7	39	7	73	7	14
8	7	8	37	8	71	8	13
9	5	9	36	9	70	9	1
17 0	04	0	634	7 0	569	3 0	511
1	03	1	33	1	68	1	10
	0		3		67		09
3	00	3	31	3	65	3	08
4	699	4	30	4	64	4	07
	69	5	8	5	63	5	05
6	96	6	6	6	6	6	04
	94	7	24		61	7	03
8	93	8		8	59	8	0
9	91	9	1	9	58	9	01
18 0	690	3 0	6 0	8 0	55	33 0	.2500
1	88	1	19	1	56	1	499
	87		17	2	55	2	98
3	86	3	16	3	53	3	97
4	84	4	1	4	5	4	96
	82	5	14	5	51	5	94
6	81		1	6	50	6	93
	9	7	11	7	49	7	9
8	78	8	10	8	4	8	91
9	76	9	08	9	46	9	90
19 0	675	4 0	60	9 0	54	34 0	459
1	74	1	06	1	44		
	73		04	2	43		
3	7	3	03	3	41		
4	71	4	0	4	40		
5	69	5	01	5	39		
6	68	6	599	6	38		
7	67	7	98		37		
8	66	8	97	8	35		
9	64	9	95	9	34		

9 Take leveling bulb from lower support and create a vacuum in the mixing chamber by lowering the bulb below the lower support When the blood mixture reaches the 50 ml mark close the vacuum controlling stopcock. Place leveling bulb on lower support and shake for three minutes
10 Raise the contents of the chamber by opening the vacuum controlling stopcock at a fairly rapid rate to prevent the re absorption of the carbon dioxide Stop when the gas pha e reaches the 2 ml mark and read immediately (P) If you overshoot the 2 ml mark draw back to the 50 ml mark and shake again for three minutes Read again on the 2 ml mark
11 Drop gas phase to approximately 5 ml region of the chamber by creating a vacuum again
12 Add approx 1 5 ml of air free NaOH to the cup By opening the main stopcock admit drop by drop one ml of NaOH
13 Seal the main stopcock again with mercury and draw off the excess NaOH in the cup by mean of a vacuum
14 Allow to stand one minute for complete absorption of the carbon dioxide and adjust the gas phase to the 2 ml mark. This is done by opening the vacuum con trolling stopcock slowly If the mark is overshot just lower the gas phase below the 2 ml mark allow to drain again and read (P) Release pressure and place leveling bulb on upper support Expel the blood mixture into the waste jar Place the leveling bulb at the lower support and clean the apparatus again with lactic acid and water
15 Record temperature of water jacket

Calculation

ml total CO_2/100 ml blood =
($P_1 - P - C$) × temperature factor for CO from Table 17

Example

P 271 3 mm P ,0 0 mm C 1 3 mm
Water jacket temperatume 25 C
ml total CO_2/100 ml blood =
(.71 3 − 70 0 − 1 3) × 0 °594 = 51 9 ml/100 ml

Precautions

See section on apparatus

6 Oxygen and Carbon Dioxide in Single Samples of Blood (Van Slyke)

Reference

Van Slyke D D and Neill J M The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurements VIII. Combined Determination of Oxygen and Carbon Dioxide in Blood J Biol. Chem. 61 561 562 (Sept) 19°4

Principle

By combining the technique for oxygen alone and carbon dioxide alone a s ngle sample suffices for both substances In our experience the most consistent results are obtained when the size of samples is 0 5 ml

Apparatus

The Van Slyke manometric apparatus

Reagents

1 Caprylic alcohol (Eastman Kodak Co)
2 Combined reagent Mix equal volumes of the following
a Lactic acid 1 2°' in water

b Ferricyanide solution containing in 1000 ml water 32 gm potassium ferricyanide [K Fe(CN)] 8 gm pure saponin and 3 ml caprylic alcohol. Shake before mixing with lactic acid solution

3 Sodium hydroxide 1 N Dissolve 40 gm in 1000 ml of water

4 Sodium hydrosulfite See the Oxygen method.

Procedure

1 Rinse out apparatus with lactic acid and water before proceeding with the an alys s Admit drops of caprylic alcohol into capillary of main (entry) cock

2 Admit 6 ml combined reagent to vacuum chamber Seal with mercury Evacu ate *Shake for 3 minutes*

3 Expel air and let out reagent to 5 ml mark in cup

4 Take in exactly 0 5 ml blood and lower reagent in cup to 3 0 ml mark
If using 1 0 ml blood lower reagent to 3 5 ml mark

5 Seal chamber and suck off excess fluid from cup Evacuate to 50 ml mark and shake for three minutes

7 Adjust gas phase to 2 ml mark in chamber

8 Read P value on mercury manometer If mark is overshot evacuate and shake *for three minutes more*

9 Lower contents to 5 ml region Add 1 0 ml NaOH to cup and admit 0 5 ml drop by drop into chamber

10 Seal with mercury Wait a few seconds while CO is absorbed.

11 Adjust gas phase to 2 ml mark and read P value Recheck P by lowering to approx 5 ml and adjusting to the 2 ml mark again The reading should be constant before continuing with the next step

12 From here on proceed as in oxygen method. The final reading P = pressure of gaseous N

13 Record the temperature in the water jacket

Calculation (0.5 ml samples)

1 ml CO_2/100 ml blood =
(P − P − C_{CO_2}) × (temp factor for CO Table 17) × [illegible]

2 ml total O_2/100 ml blood =
(P − P − C_O) × (temp factor for O_2, Table 16) × 2

Example

Size of blood sample 0 5 ml
P 193 0 mm P 108 mm. P_a 0 0 mm
Water Jacket 5 C C for CO_2 1 3 C for O 0 7
ml CO_2/100 ml blood =
(193 0 − 108 − 1.3) × 0 594 × 2 = 45 9
ml O_2/100 ml blood =
(108.2 − 0 0 − 0 7) × 0.2406 × 2 = 18 0

Precautions

See section on apparatus. We have obtained more reproducible results using 0 5 ml samples than when using 1 ml samples

Oxygen Capacity (Van Slyke)

Reference

Roughton F J W Darling R. C and Root W S Factors Affecting the Determination of Oxygen Capac ty Content and Pres ure in Human Arterial Blood Am J Physiol. 142 03 0 (Dec) 1944

9 Take leveling bulb from lower support and create a vacuum in the mixing chamber by lowering the bulb below the lower support When the blood mixture reaches the 50 ml mark close the vacuum controlling stopcock Place leveling bulb on lower support and shake for three minutes
10 Raise the contents of the chamber by opening the vacuum controlling stopcock at a fairly rapid rate to prevent the re absorption of the carbon dioxide Stop when the gas phase reaches the 2 ml mark and read immediately (P) If you overshoot the 2 ml mark draw back to the 50 ml mark and shake again for three minutes Read again on the 2 ml mark
11 Drop gas phase to approximately 5 ml region of the chamber by creating a vacuum again
12 Add approx 1 5 ml of air free NaOH to the cup By opening the main stopcock admit drop by drop one ml of NaOH
13 Seal the main stopcock again with mercury and draw off the excess NaOH in the cup by mean of a vacuum
14 Allow to stand one minute for complete absorption of the carbon dioxide and adjust the gas phase to the 2 ml mark This is done by opening the vacuum controlling stopcock slowly If the mark is overshot just lower the gas phase below the 2 ml mark allow to drain again and read (P) Release pressure and place leveling bulb on upper support Expel the blood mixture into the waste jar Place the leveling bulb at the lower support and clean the apparatus again with lactic acid and water
15 Record temperature of water jacket

Calculation

ml total CO_2/100 ml blood =
(P_1 – P – C) × temperature factor for CO from Table 17

Example

P 271 3 mm P 70 0 mm C 1 3 mm
Water jacket temperatume 25 C
ml total CO_2/100 ml blood =
(271 3 – 70 0 – 1 3) × 0 2594 = 51 9 ml/100 ml

Precautions

See section on apparatus

6 Oxygen and Carbon Dioxide in Single Samples of Blood (Van Slyke)

Reference

Van Slyke D D and Neill J M The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurements VIII. Combined Determination of Oxygen and Carbon Dioxide in Blood J Biol Chem *61 561 562 (Sept) 1924*

Principle

By combining the technique for oxygen alone and carbon dioxide alone a single sample suffices for both substances In our experience the most consistent results are obtained when the size of samples is 0 5 ml

Apparatus

The Van Slyke manometric apparatus

Reagents

1 Caprylic alcohol (Eastman Kodak Co)
2 Combined reagent Mix equal volumes of the following
a. Lactic acid 1 2% in water

b Ferricyanide solution containing in 1000 ml water 3 gm potassium ferricyanide [$K_3Fe(CN)_6$] 6 gm pure saponin and 3 ml caprylic alcohol. Shake before mixing with lactic acid solution.
3 Sodium hydroxide 1 N Dissolve 40 gm in 1000 ml of water
4 Sodium hydrosulfite See the Oxygen method.

Procedure

1 Rinse out apparatus with lactic acid and water before proceeding with the analysis. Admit 2 drops of caprylic alcohol into capillary of main (entry) cock.
2 Admit 6 ml combined reagent to vacuum chamber Seal with mercury Evacuate Shake for 3 minutes
3 Expel air and let out reagent to 5 ml mark in cup
4 Take in exactly 0 5 ml blood and lower reagent in cup to 3 0 ml mark
5 If using 1 0 ml blood lower reagent to 3 5 ml mark.
6 Seal chamber and suck off excess fluid from cup Evacuate to 50 ml mark and shake for three minutes
7 Adjust gas phase to ml mark in chamber
8 Read P value on mercury manometer *If mark is overshot evacuate and shake for three minutes more*
9 Lower contents to 5 ml reg on Add 1 0 ml NaOH to cup and admit 0 5 ml drop by drop into chamber
10 Seal with mercury Wait a few seconds while CO is absorbed
11 Adjust gas phase to 2 ml mark and read P value Recheck P by lowering to approx 5 ml and adjusting to the ml mark again The reading should be constant before continuing with the next step
1 From here on proceed as in oxygen method. The final reading P_4 = pressure of gaseous N_2.
13 Record the temperature in the water jacket

Calculation (0.5 ml samples)

1 ml CO_2/100 ml blood =

$(P - P - C_{CO}) \times$ (temp factor for CO Table 17) $\times 2$

2 ml total O_2/100 ml blood =

$(P - P - C_O) \times$ (temp factor for O Table 16) $\times 2$

Example

Size of blood sample 0 5 ml.
P_1 198 0 mm P 108 mm. P_4 3 0 mm
Water Jacket C C for CO 1 3 C for O 0
ml CO_2/100 ml blood =
(198 0 - 108 - 1 3) × 0 94 × 2 = 45 9
ml O_2/100 ml blood =
(108.2 - 0 0 - 0 7) × 0 406 × 2 = 18 05

Precautions

See section on apparatus We have obtained more reproducible results using 0.5 ml samples than when using 1 ml samples

7 Oxygen Capacity (Van Slyke)

Reference

Roughton F J W Darling R. C. and Root, W S Factors Affecting the Determination of Oxygen Capacity Content and Pressure in Human Arterial Blood Am J Physiol 14 08 0 (Dec) 1944

9 Take leveling bulb from lower support and create a vacuum in the mixing chamber by lowering the bulb below the lower support When the blood mixture reaches the 50 ml mark close the vacuum controlling stopcock Place leveling bulb on lower support and shake for three minutes

10 Raise the contents of the chamber by opening the vacuum controlling stopcock at a fairly rapid rate to prevent the re absorption of the carbon dioxide Stop when the gas pha e reaches the 2 ml mark and read immediately (P) If you overshoot the 2 ml mark draw back to the 50 ml mark and shake again for three minutes Read again on the 2 ml mark

11 Drop gas phase to approximately 5 ml region of the chamber by creating a vacuum again

12 Add approx 1 5 ml of air free NaOH to the cup By opening the main stopcock admit drop by drop one ml of NaOH.

13 Seal the main stopcock again with mercury and draw off the excess NaOH in the cup by mean of a vacuum

14 Allow to stand one minute for complete absorption of the carbon dioxide and adjust the gas phase to the 2 ml mark This is done by opening the vacuum controlling stopcock slowly If the mark is overshot just lower the gas phase below the 2 ml mark allow to drain again and read (P) Release pressure and place leveling bulb on upper support Expel the blood mixture into the waste jar Place the leveling bulb at the lower support and clean the apparatus again with lactic acid and water

15 Record temperature of water jacket

Calculation

ml total CO_2/100 ml blood =
(P − P − C) × temperature factor for CO from Table 17

Example

P 271 3 mm P 70 0 mm C 1 3 mm
Water jacket temperatume °5 C
ml total CO_2/100 ml blood =
(271 3 − 70 0 − 1 3) × 0 2594 = 51 9 ml/100 ml

Precautions

See section on apparatus

6 Oxygen and Carbon Dioxide in Single Samples of Blood (Van Slyke)

Reference

Van Slyke D D and Neill, J M The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurements VIII. Combined Determination of Oxygen and Carbon Dioxide in Blood J Biol Chem. 61 561 56? (Sept) 19–4

Principle

By combining the technique for oxygen alone and carbon dioxide alone a single sample suffices for both substances In our experience the most consistent results are obtained when the size of samples is 0 5 ml

Apparatus

The Van Slyke manometric apparatus

Reagents

1 Caprylic alcohol (Eastman Kodak Co)
2 Combined reagent Mix equal volumes of the following
a. Lactic acid 1 2% in water

b Ferricyanide solution containing in 1000 ml water 3 gm potassium ferricyanide [$K_3Fe(CN)_6$] 8 gm pure saponin and 3 ml caprylic alcohol Shake before mixing with lactic acid solution.

3 Sodium hydroxide 1 N Dissolve 40 gm in 1000 ml of water

4 Sodium hydrosulfite See the *Oxygen method*

Procedure

1 Rinse out apparatus with lactic acid and water before proceeding with the analysis. *Admit 2 drops of caprylic alcohol into capillary of main (entry) cock.*

2 Admit 6 ml combined reagent to vacuum chamber Seal with mercury Evacuate Shake for 3 minutes.

3 Expel air and let out reagent to 5 ml mark in cup

4 Take in exactly 0 5 ml blood and lower reagent in cup to 3 0 ml mark

5 *If using 1 0 ml blood lower reagent to 3 5 ml mark*

6 Seal chamber and suck off excess fluid from cup Evacuate to 50 ml mark and shake for three minutes

7 Adjust gas phase to 2 ml mark in chamber

8 Read P value on mercury manometer If mark is overshot evacuate and shake *for three minutes more*

9 Lower contents to 5 ml region. Add 1 0 ml NaOH to cup and admit 0 5 ml drop by drop into chamber

10 Seal with mercury Wait a few seconds while CO is absorbed.

11 Adjust gas phase to ml mark and read P value Recheck P by lowering to approx 5 ml and adjusting to the 2 ml mark again The reading should be constant before continuing with the next step

12 From here on proceed as in oxygen method. The final reading P = pressure of gaseous N_2.

13 Record the temperature in the water jacket

Calculation (0.5 ml samples)

1 ml CO_2/100 ml blood =

$(P - P - C_{CO}) \times$ (temp factor for CO, Table 17) $\times 2$

2 *ml total O_2/100 ml blood* =

$(P - P - C_O) \times$ (temp factor for O Table 16) $\times 2$

Example

Size of blood sample 0 5 ml.

P_0 198 0 mm P 108.. mm P_N 0 0 mm

Water Jacket 5 C. C for CO 1 3 C for O 0

ml CO_2/100 ml blood =

$(198\ 0 - 108\ 2 - 1\ 3) \times 0\ 394 \times 2 = 43\ 9$

ml O_2/100 ml blood =

$(108.2 - 0\ 0 - 0\ 7) \times 0\ {}^{\circ}400 \times {}^{\circ} = 18\ 0.$

Precautions

See section on apparatus. We have obtained more reproducible results using 0 5 ml samples than when using 1 ml samples.

7 Oxygen Capacity (Van Slyke)

Reference

Roughton F J W Darling R. C. and Root W S *Factors Affecting the Determination of Oxygen Capacity Content and Pressure in Human Arterial Blood* Am J Physiol 142 08 0 (Dec) 1944

Principle

The reference cited discusses significant errors in the common technique for determining oxygen capacity, i e equilibrating blood with air *in a tonometer* *The* authors recommend equilibration directly in the Van Slyke extraction chamber

Apparatus

1 Van Slyke manometric apparatus
2 Roughton Scholander syringe pipette

Reagents

1 *Sodium chloride solution, 0 9%*, equilibrated in a tonometer with 100 per cent oxygen
2 A solution of 3 % potassium ferricyanide and 8% of saponin deaerated in a tonometer and transferred to a burette (no oil) with no foam
3 Sodium hydroxide 0 1 N Deaerate and store under oil
4 Pyrogallic acid 15% in sodium hydroxide solution (20%)
5 Caprylic alcohol
6 Clean mercury

Procedure

1 Clean chamber as in previous analyses
2 Admit two drops of caprylic alcohol and 1 ml of oxygenated saline into the chamber
3 Add exactly 1 ml blood into the cup and wash into the chamber with 5 ml of oxygenated saline
4 Mix contents of chamber and let in room air until mercury level is at the 50 ml mark.
5 Shake for two minutes and expel all the air quantitatively *Note that this is the* time at which the blood is saturated with oxygen.
6 Add 0 25 ml of ferricyanide saponin solution and seal the chamber with mercury
7 Lower the mercury level to the 50 ml mark and shake the chamber for three minutes
8 Pelease the pressure of the gas phase to approximately 5 to 10 ml
9 Add 1 5 ml of 0 1 N NaOH in the cup and admit 1 ml into the chamber slowly Seal with mercury and read at the 2 ml mark (P) When reading is constant proceed with the analyses
10 Add 1 5 ml of pyrogallic acid solution to the cup and admit 1 ml *very slowly* keeping liquid level near the 2 ml mark Allow two to three minutes for the absorption
11 *Before sealing mix contents in the chamber open lower stopcock* allowing gas to contract to a small bubble seal slowly so that last oxygen is absorbed by the last few drops of pyrogallol
12 Lower just below the two ml mark allow to drain and read (P) at the 2 ml mark

Calculation

See Oxygen in blood (Van Slyke)

Example

See Oxygen in blood (Van Slyke)

Precautions

1 The oxygen capacity of arterial blood as obtained by 15 minutes saturation with *air in a tonometer followed* by analysis in the Van Slyke Neill manometric apparatus is considered to be about 2 per cent higher than the value in the blood at the moment of withdrawal from the blod vessel Three factors contribute to this discrepancy

a Drainage errors in the saturator These cause the blood sample removed for analysis to be unduly rich in red cells (average effect equals about 0 7 per cent)

b Gradual reversion of some of the inactive pigment to the active gas combining form. This effect is very variable but on the average equals 1 0 to 1.5 per cent of the total capacity Owing to the smallness of the effect the nature of the inactive reverting pigment has not been established. It may be in part methemoglobin

c Presence of traces of COHb in the blood.

9 Correction for these factors raises the average percent O Hb of normal man at sea level from 95 to 97 the arterial pO as calculated from these figures and the blood dissociation curves comes out to 100 mm in agreement with alveolar pO rather than to the average figure of 80 mm given in recent papers The latter figure is believed to be false

8 Carbon Monoxide in Blood (Total Heme Pigments)

Reference

Horvath S M and Roughton F J W Improvements in the Gasometric Estimation of Carbon Monoxide in Blood J Biol. Chem 144 747 755 (June) 194

Principle

Dissolved carbon monoxide dissolved nitrogen and dissolved carbon d oxide are liberated into the gas phase of the Van Slyke manometri apparatus Bound carbon monoxide and carbon dioxide are releas d at pH 6 by shaking with a hydrosulfite ferricyanide solution Carbon dioxide is absorbed with sodium hydroxide and the residual gas is solely carbon monoxide

Apparatus

1 Van Slyke Neill manometric apparatus
2 Roughton Scholander syringe pipette

Reagents

1 Caprylic alcohol (Eastman Kodak Co)
2 Saponin a 1% solution of pure white saponin
3 Sod um hydrosulfite ($Na_2S_2O_4$) a 2% solution in saturated sodium borate A stock of 3% sodium borate ($Na_2B_4O_7$ $2H_2O$) is prepared. At the beginning of the day 50 ml are placed in a 50 ml Erlenmeyer flask 1 gm of solid $Na_2S_2O_4$ is dropped in the flask is quickly corked and shak n w th only a minute bubble of air therein. The solution is at once transferred to a 50 ml burette
4. Potassium ferricyanide [$K_3Fe(CN)_6$] Prepare a 30% air free solution by shaking in an evacuated tonometer and transferring to a burette with rubber outlet and pinchcock. If it darkens a fresh supply must be made.
5 Acetate buffer 70 gm of sodium acetate plus 100 ml water and 15 ml of glacial acetic acid.
6 Mixed acetate buffer and $K_3Fe(CN)_6$ reagent (air free) 50 ml of $K_3Fe(CN)_6$ solution plus 1 5 ml of the acetate buffer
7 Sodium hydroxide (NaOH) a 10% solution (air free)
8 Water saturated with carbon monoxide at one atmosphere. Keep under paraffin oil in a 50 ml burette
9 Clean mercury (See oxygen method)

Procedure

1. Admit 5 ml of water into a clean chamber seal with mercury evacuate and shake for _ minutes.

2 Expel all the water into the waste jar and seal off both ends of the main stop cock with mercury
3 5 drops of caprylic alcohol are drawn into the extracting chamber
4 Place 1.5 ml of 1% saponin solution into the cup
5 Exactly 1 or 2 ml of blood (according to whether the per cent COHb is greater or less than 50) are pipetted directly into the chamber followed by the remainder of the saponin solution that is in the cup
6 Mix in chamber by raising and lowering the leveling bulb, and allow one minute for complete laking
7 Place 2 ml of the hydrosulfite borate solution into the cup and draw 1.5 ml into the chamber
8 Mix again and allow to stand for one minute
9 Draw off and discard the excess hydrosulfite borate from the cup and add 18 ml of the carbon monoxide water into the chamber
10 Seal off with mercury and lower the mercury level in the chamber to the 50 ml mark
11 Cover the chamber with black paper and shake the solution for two minutes.
12 The evolved gases are quantitatively ejected. Seal again with mercury and shake again for two minutes more Expel gases again (O_2 and N_2) and remove paper from chamber
13 Place 3.0 ml of air free ferricyanide buffer solution into the cup and take ?.0 ml into the chamber
14 Seal with mercury and discard remaining buffer $K_3Fe(CN)_6$ solution
15 Lower the mercury level to approximately 1 cm below the 50 ml mark and shake the mixture for a total of 5 minutes
16 Once during the shaking the motor is temporarily stopped and the mercury raised to the 50 ml mark, so as to mix the solution in the stem of the chamber with the main body of the solution In this way destruction of the ferricyanide by the mercury is minimized
17 At the end of 5 minutes the gas is then compressed nearly to atmospheric pressure and 2.5 ml of the air free NaOH placed in the cup
18 Take 1.5 ml of the NaOH into the chamber and allow about one minute for complete carbon dioxide absorption Try not to let the solution get above the 2 ml mark
19 Adjust the solution meniscus to the 2 ml mark and read the pressure (P_1)
20 Using a Roughton Scholander syringe pipette quantitatively eject the gas sample into it for CO analysis by the technique described in the sections on Roughton Scholander methods
21 Lower the solution below the 2 ml mark allow to drain and read again at the ml mark (P_2)
22 Record temperature of the water jacket

Calculation

1 ml total CO/100 ml blood =
$(P_1 - P_2 - C) \times$ temp factor for O_2 Table 16
2 The correction for CO is calculated from the analysis of the sample in the syringe pipette

Example

One ml of blood was analyzed from a tonometer in which the blood was saturated with CO P_1 was 134.2 P_2 was 47.8 C was 0.5 Water jacket was at 21.6 C
ml total CO/100 ml blood =
$(134.2 - 47.8 - 0.5) \times 0.2445 = 20.84$ ml CO/100 ml blood

Precautions

1 Be sure to cover the chamber with black paper as light will destroy carbon monoxide heme complexes

Oxygen in Blood (Roughton Scholander)

Reference

Roughton F J W and Scholander, P F Micro Gasometric Estimation of the Blood Gases I Oxygen J Biol Chem. 148 541 550 (June) 1943

Principle

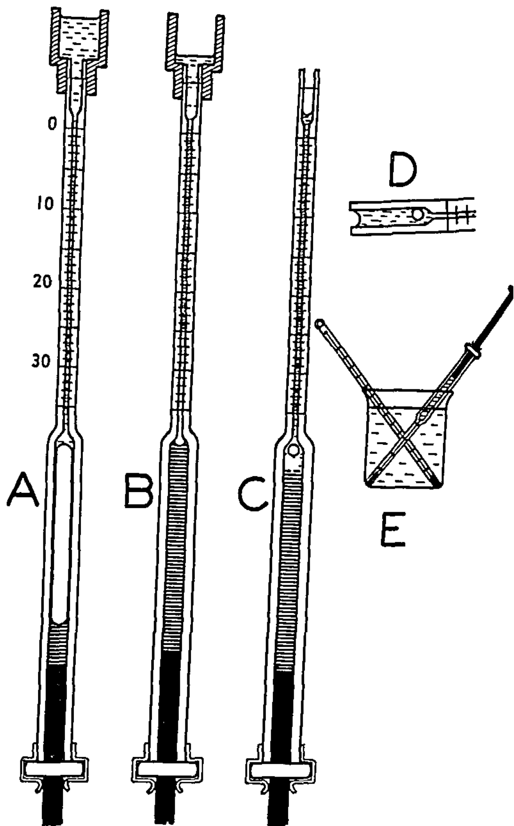
By mixing of blood in a spec al syringe with ferricyanide containing potassium bicarbonate and saponin and adding an acetate buffer a gas phase is provided for the extraction of the blood gases in the same way the vacuum acts in the Van Slyke method The CO thus produced is then absorbed with 10 percent NaOH The residual gas bubble is driven into the syringe s cap llary and its volume measured before and after absorption of O with alkaline pyrogallol. The difference in these two volumes represents the O content of the blood and reagents Then a blank is run for the O content of the reagents alone this figure is subtracted from the former and the rema nder when multipl ed by the usual correction factor for tem perature and pressure gives the O content of the blood

Apparatus

1 A one ml Pyrex tuberculin syringe with arresting cl p and with standard bore precision 0 5 mm Pyrex capillary fused to its nozzle The top of the capillary is expanded to a cylindrical cup of about 2 5 mm bore and 1 5 mm length The capillary (7 to 8 cm length) is graduated into 30 division each of 2 mm length (Fig 9)
2 Blood pipette made of thin walled glass tubing (1 to 1 5 mm bore) with a tip to fit into the bottom of the gla s cup The volume delivered from the mark to the tip is equal to 100 d visions of th capillary and is actually 39 3 cmm
3 A detachable rubber cup of about 1 ml capacity is fitted to the top of the glass cup when req ired.
4 Several 2 ml and 5 ml syringes with fine t pped rubber connected gla s nozzle to store the reagents
5 Metal or glass beakers one of them contain ng water at room temp rature to place the syringe for equilibration between readings
6 Suction line and fi e tipped glass point
7 When suction system is not available a regular syringe or dropper can be used.
8 Lens paper or a piece of cloth to wipe the cap llary between readings
9 A piece of fine wire to remove air bubbles from the cup
10 Thermometer for water bath

Reagents

1 Distilled water
2 Caprylic alcohol
3 Ferricyanide solution 1 5 gm of $K_3Fe(CN)$ 3 gm of KHCO and 1 5 gm of saponin are dissolved in water and made up to 50 ml It is not adv sable to make larger amounts as the solution should be renewed every 3 days.
4. Acetate buffer 70 gm of sodium acetate NaC_2H_3O 3H O are dissolved in 100 ml of water and 15 ml of glacial acetic acid are added.
5 Urea 45 percent This is used as a cleaning solution because of its protein dissolving property
6 Sodium hydroxide 10 per cent.
7 Pyrogallol solution. 15 gm of powdered pyrogallol are ad led to 100 ml of 20 percent NaOH in a rubber stoppered bottle and covered with a layer of oil – cm thick The pyrogallol is dissolved under the oil by stirring with a glass rod.
8 The di tilled water is placed in a bottle above the apparatus and is delivered through rubber tubing with a glass nozzle It can also be delivered by a 20 ml syringe with a needle or a glass nozzle.



Figs 29 31

Fig 29 (*A B C*) —Technique for absorption of excess of CO_2

Fig 30 (*D*) —Technique for absorption of CO (or other absorbable gas) in glass cup

Fig 31 (*E*) —Temperature equilibration of gas bubble in capillary

9 Reagents 2 3 4 5 and 7 are conveniently stored in ⌄ or 5 ml syringes and Reagent 6 in a 10 or 20 ml syringe These syringes have fine tipped glass nozzles at tached.

Procedure

1 The syringe is held vert cally and any liquid in the cup of the syringe is with drawn by suction The plunger is pushed up and the cup filled with ferricyanide solution. The solution is drawn down to the bottom of the syringe and ex pelled through the cup and removed. This procedure is repeated twice with fresh lots of ferricyanide without trapping air bubbles No grease or oil is used in the syringe

2 The glass cup is filled to the mark with ferricyanide and the latter drawn down to the bottom of the cup

3 A drop of caprylic alcohol is deposited on the bottom of the cup

4. The pipette is filled with blood to the mark wiped and cautiously introduced into the glass cup and its tip pressed snugly but not too vigorously against the bottom of the cup The pipette should be held at a slight angle to the horizontal so that the blood does not run out when both ends of the pipette are open to the air (see Figs 30 31 3 and 33)

5 By pulling out the plunger gradually the blood is slowly and evenly drawn down into the capillary followed by a bubble of air of about 1 mm length No ap preciable amount of caprylic alcohol should be drawn in during this step The bubble of air prevents any blood being sucked back when the p pette is re moved (See Fig 33 *B*)

6 The p pette is quickly removed and the bubble of air is then expelled through the caprylic alcohol with the aid of the wire

7 A trace of capryl c alcohol i e about two divisions length of the capillary is drawn down onto the top of the bood and the rest of the caprylic alcohol is sucked out from the cup

8 The cup is filled to the mark w th acetate buffer and the latter drawn down to the bottom of the cup

9 The cup is then immediately filled to the top with 45 percent urea solution and then closed firmly with the finger

10 The arresting clip is then removed and the closed apparatus v gorously shaken in the horizontal position The plunger will be gradually drawn out by itself as the CO and other gases are evolved the gas pressure in the syringe being kept roughly atmo pheric The total volume evolved is usually about 0 ⌄ ml. Shak i g is continued for a total of two minutes (see Figs 33 and 34)

11 The arre ting clip is put back in place

1 The finger is cautiously released the gas meniscus being kept in the capillary by man pulating the plunger

13 A small amount of urea is allowed to run down into the capillary and left there until the walls are perfectly clean

14 Three quarters of the urea solution in the glass cup is removed by gentle suc tion

15 *The rubber cup is adjusted to the cup and filled with 10 per cent* NaOH without trapping air bubble Use the fine wire if necessary

16 A little NaOH is drawn into the syringe. This absorbs some CO causing a partial va uum which quickly sucks in more NaOH until only a small bubble consist ng of O_2 N_2 and CO (if any was originally present in the blood) is left at the top of the syringe. The absorption takes a few seconds and just before it is complete the residual bubble is screwed slowly and carefully up into the capillary by manipulation of the plunger

17 The rubber cup is removed As it st ll contains some NaOH it is advisable to remove the cup under a stream of running water The glass cup is then emptied by suction.

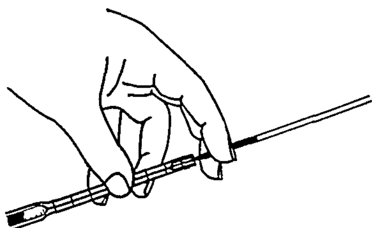


Fig 3 —Transfer of blood direct into capillary

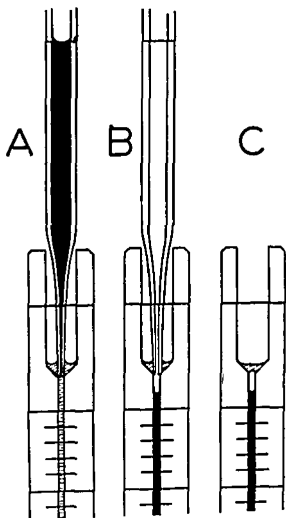


Fig 33 (*A B C*) —Further details of transfer of blood into capillary

18 The capillary is placed for half a minute in a beaker of water at room temperature and the temperature read (Fig 31)

19 It is then removed dried by light wiping care being taken that the capillary is not handled and the volume of the bubble read = V divisions.

20 The glass cup is then filled with pyrogallol solution and the O of the bubble is absorbed by pulling the gas bubble down to the bottom of the capillary and back again a few times Finally the bubble is moved very slowly up into the top part of the capillary and after a further temperature equilibration its volume is read again V division If V_2 is only a few divisions the second temperature equilibration can be omitted

21 A blank is obtained by following exactly the same steps except the adding of the blood sample

Calculations

The oxygen content of the blood equals

$(V - V - c) \times f$ where

V First reading after absorption of the CO with NaOH

V Second reading after absorption of the O with pyrogallol

c Blank correction for O content of reagents

f Correction factor for temperature aqueous vapor pressure and barometric pressure as derived from the line chart at the beginning of th section on blood gases (Fig 5)

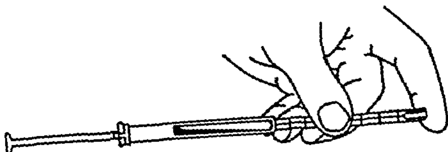


Fig 34—Shaking of syringe and extraction of gas.

Example

First reading	27 8	(V)
Second reading	5.5	(V)
Blank figure	1 1	(c)
Correction factor	0 900	(f) for T = 2 C and bar pre s = 60 mm

27 8 − 5 5 − 1 1 multipl ed by 0 900 = 21.2 × 0 9 = 19 1 ml of O per 100 ml blood

Precautions

1 When blood is drawn from the pipette into the syringe care should be taken that the pipette be pressed snugly but not too vigorously against the bottom of the cup in order to prevent breakage of the tip

2 The arresting cl p taken off at the beginning of the shaking should be put back in place before releasing finger

3 Speedy adjustment of the bubble into the capillary at the end of th CO absorption helps to eliminate reab orption of O at the critical moment at which the partial pressure of the O_2 becomes high (i.e nearly 1 atmosphere)

4 While there is gas in the syringe or bubbles in the capillary the hands should not touch either one so as to prevent any effect of the hands warmth on the volume of the gases.

5 Slipping back of the plunger is prevented by a proper adjustment of the arresting clip
6 Temperature equilibration of the bubble *before the measurement of* V has proved necessary for with long bubbles a contraction of nearly a division may be observed as a result of immersion of the capillary in the water bath
7 To wash the instrument the plunger is pulled out under a stream of running water and the blood mixture poured out The syring is filled and emptied several times with water before the plunger is restored The plunger should never be forced inwards if there is a resistance due to precipitates inside it Occasionally the whole syringe should be rinsed with dichromate cleaning solution.

10 Dissolved Nitrogen in Blood (Edwards Scholander Roughton)

Reference

Edwards G A Scholander P F and Roughton F J W Micro Gasometric Estimation of the Blood Gases III Nitrogen J Biol Chem 148 565 571 (June) 1943

Principle

A CO_2 phase is evolved by mixing the blood sample with bicarbonate and acid phosphate and thus the whole of the dissolved nitrogen of the blood and reagents is extracted as well as some oxygen No CO is extracted The CO and O are then absorbed with alkaline hydrosulfite and the bubble of N which is left is measured in the syringe capillary The reading less a small blank from the reagents is multiplied by the usual correction factor for temperature and pressure and the figure so obtained gives the N_2 content of the blood in volumes percent

Apparatus

1 One ml Pyrex tuberculin syringe with arresting clip and with a standard bore precision 0 5 mm Pyrex capillary fused to its nozzle The top of the capillary is expanded to a cylindrical cup of about 2 5 mm bore and 1 5 cm length The capillary (7 to 8 cm length) is graduated into 30 divisions each of ° mm length
2 Blood pipette made of thin walled glass tubing (1 to 1 5 mm bore) with a tip to fit into the bottom of the glass cup The pipette has two marks one near the tip where it is calibrated to deliver an amount equal to 100 divisions of the capillary and which actually is 39 3 cmm The other mark is calibrated to contain thrice as much as the former amount (See Figs 29 34)
3 A detachable rubber cup of about 1 ml capacity is fitted to the top of the glass cup when required.
4 Several 2 ml and 5 ml syringes with fine-tipped rubber connected glass nozzles to store the reagents.
5 Metal or glass beakers one of them containing water at room temperature to place the syringe for equilibration between readings
6 Suction line and fine tipped glass point
7 A regular syringe or a dropper when suction system is not available
8 Lens paper or a piece of cloth to wipe the capillary before readings
9 A piece of fine wire to remove air bubbles from the cup
10 Thermometer for water bath

Reagents

1 Aerated distilled water
2 Caprylic alcohol
3 Bicarbonate solution 11 gm of KHCO are dissolved in 100 gm of water
4 Acid phosphate buffer 95 gm of $NaH_2PO_4 \cdot H_2O$ are dissolved in 100 gm of warm water The resultant volume is about 142 ml at 0 C and the strength about 5 M
5 Urea 45 percent in water

6 Hydrosulfite solution 15 gm of a mixture of sodium hydrosulfite 10 parts and sodium anthraquinone beta sulfonate 1 part are added to 50 ml of 20 percent KOH This solution should be made with the least possible contamination from air and stored in a rubber stoppered bottle

7 Reagents ° to 4 are stored in well greased ml syringes provided with glass tips Reagents ʋ and C may be stored in 10 ml syringes provided with glass tips The distilled water may best be stored in a bottle with rubber tubing and glass nozzle hanging down over the apparatus A syringe may serve for this purpose as well.

Procedure

1 The syringe is held vertically and any liquid in the cup of the syringe is with drawn The plunger is pushed up and the syringe cup filled with bicarbonate solution The solution is then drawn down into the syringe barrel the plunger being pulled practically the whole way out The plunger is then pushed up without any bubbles being trapped and the b carbonate withdrawn from the syringe cup The step is repeated once more The dead space in the syringe nor mally contains enough b carbonate to give 0 ˜5 ml of CO on subsequent treat ment with the acid buffer

° The walls of the syringe cup are dried with a roll of cotton or cleaning paper and a drop of capryl c alcohol placed in the bottom of the cup without trapping any air bubbles

3 The blood is measured from the second mark of the p pette into the capillary under the caprylic alcohol With the syringe at the same angle the pipette is cautiously introdu ed into the glass cup and its t p pressed snugly but not too vigorously against the bottom of the cup

4 The bloo l is drawn down into the syringe until the upper end of the column of blood is ° mm below the top of the syringe capillary with a small bubble of air above it Care must be taken that no caprylic alcohol is drawn down with the blood The pipette is then removed the air bubble is expelled through the alco hol by gently screwing up the plunger and 2 divisions of caprylic alcohol are left on top of the blood in the capillary

5 The cup is filled to the mark with the acid buffer and the latter drawn down to the bottom of the cup

6 The cup is filled to the top with urea solution and is closed firmly with the finger

7 The arresting clip is taken off the syringe barrel

8 The syringe is shaken vigorously for ° minutes in the horizontal position the plunger being withdrawn as the CO_2 is evolved The gas pressure in the syringe must be kept at atmospheric pressure. The volume of the gas finally evolved must be between 0 6 and 0 5 ml.

9 The arresting clip is put back in place and then the finger tip is cautiously re leased the plunger being manipulated so as to keep the gas meniscus in the capillary

10 The urea solution is drawn down to the bottom of the capillary without being allowed to enter the syringe barrel. This prevents sub equent contamination of the capillary wall with precipitates from the blood.

11 The syringe is held in the vertical position the rubber cup attached and about 1 ml of the hydrosulfite solution added without trapping air bubbles in the syringe cup

1 A little of the hydrosulfite is gently drawn down into the syringe creating a vacuum which sucks in the rest of the solution needed for absorption of the CO_2 and such traces of O_2 as are present.

13 The bubble is pushed up into the lower part of the cap llary The hydrosulfite is sucked out of the rubber cup and the latter detached under a stream of runn ng

water The syringe cup is then filled with water three fourths of which is drawn down over the bubble layering itself on top of the blood mixture

14 The bubble is pushed up into the clean capillary very gently and the capillary is placed in a beaker of water at room temperature to bring about temperature equilibrium The temperature is read.

15 The syringe is removed from the beaker, dried by light wiping care being taken that the capillary is not handled and the volume of N read V divisions

16 A blank is run to get the blank correction figure It is exactly the same procedure but no blood sample is used. Some difficulty may be encountered in first determining the blank since in the blank analysis the blood has a damping effect upon the reaction between the bicarbonate and the a id so that the CO_2 is not evolved for 10 or more seconds after the acid is drawn down into the syringe In the blank analysis on the other hand the reaction begins immediately when the acid comes in contact with the bicarbonat and hence some bubbles of gas belonging in the syringe may be lost to the outside To avoid this difficulty one must

a Make sure that the 2 divisions of caprylic alcohol are not washed out when the acid is put in the cup

b Draw the acid down into the syringe barrel quickly

c Fill the cup with urea solution immediately aft r the acid is drawn down and then clamp the finger over the glass cup at once

The blank figure has been found to be constant and of the order of 1 3 to 1.5 units on the capillary depending upon the instrument used

Calculations

The nitrogen content of the blood equals

$$\frac{(V - c) \times f}{3}$$

V = Uncorrected N_2 reading

c = Blank figure

f = Correction factor for temperature and barometric pressure (See STP chart at beginning of section on blood gases)

The Factor 3 is inserted because a volume of blood equal to 300 scale divisions is delivered in Step 3

Example

N reading = 5 2

Blank = 1 4

Corr factor 0 900 (for T = 2_ C bar press = 760 mm)

That is ml N_2/100 ml blood $= \frac{(5\,2 - 1\,4) \times 0\,900}{3} = 1\,14$

Precautions

1 When blood is drawn from the pipette into the syringe care should be taken that the pipette be pressed snugly but not too vigorously against the bottom of the cup in order to prevent the breakage of the tip

2 The arresting clip taken off at the beginning of the shaking should be put back in place before releasing finger

3 Slipping back of the plunger is prevented by a proper adjustment of the arresting clip

4 Temperature equilibration of the bubble before the measurement has proved necessary for with long bubbles a contraction of nearly a division may be observed as a result of the immersion of the capillary in the water bath

5 Great caution must be exercised in handling blood of low N content as contamination with atmospheric nitrogen occurs rapidly due to the high gradient of nearly 4/5 of an atmosphere

6 To wash the instrument the plunger is pulled out under a stream of running water and the blood mixture is poured out The syringe is filled and emptied several times with water before the plunger is restored. The plunger should never be *forced* inward if there is a resistance due to precipitate inside it. Occasionally the whole syringe should be rin ed with dichromate cleaning solution

11 Carbon Monoxide in Blood (Scholander Roughton)

Reference

Scholander P F and Roughton F J W Micro Gasometric Estimation of the Blood Ga es II Carbon Monoxide J Biol Chem 148 5.1 563 (June) 1943

(This paper describes how for the microdetermination of carbon monoxide in the blood the syringe can be used in three different ways i e

A General method for saturation ranging from 0 to 100 per cent COHb

B Method for combined determination of O and CO on *one sample of blood*

C Special method preci e enough for blood volume determination in which the CO content is kept below ° volumes per cent)

A. GENERAL METHOD FOR ESTIMATION OF CO IN BLOOD

Principle

The O CO and N of the blood and *reagents are extracted by excess of CO evolved* by the mixing of ferricyanide solution containing pota sium bicarbonate and saponin with acetate buffer The CO and O are subsequ ntly absorbed together by alkaline pyrogallol The volume of the re idual gas is measured in the capillary of the instrument before and after the absorption of CO by Winkler s solution The difference in volume multiplied by the correction factor for temp rature and pressure gives the CO content of the blood in volumes percent Since the blood *itself* is the only source of CO and the reagents contain none a blank correction is not neces ary

Apparatus

1 One ml Pyrex tuberculin syringe with arresting clip and with a standard bore precision 0 5 mm Pyrex capillary fused to its nozzle The top of the capillary is expanded to a cylindrical cup of about °.5 mm bore and 1 5 cm length. The capillary (· to 8 cm length) is graduated into 30 divisions each of ° mm length

° Blood pipette made of thin walled gla.s tubing (1 to 1.5 mm bore) with a tip to fit into the bottom of the glass cup The volume delivered from the mark to the tip is equal to 100 divisions of the capillary and is actually 39 3 cmm (See Figs. °9 34)

3 A detachable rubber cup of about 1 ml capacity is fitted to the top of the glass cup when re juired.

4 Several 2 ml and ₅ ml syringes with fine tipped rubber connected glass nozzles to store the reagents

5 Metal or glass beakers one of them containing water at room temperature to cover the syr nge for equilibrations between read ngs.

6 Suction line and fine tipped glass point.

A regul r syringe or a dropper when suction system is not ava lable

8 Cleaning paper or a piece of cloth to wipe the capillary before readings.

9 A piece of fine wire to remove air bubbles from the cup

10 A thermometer for the water bath

Reagents

1 Aerated d stilled water

° Caprylic alcohol.

3 Ferricyanide solution 12 5 gm of $K_3Fe(CN)$ 3 gm of $KHCO$ and 1.5 gm of saponin are ground in a mortar and dissolved in water and *made up to* [illegible]*0 ml*. The solution should be renewed every three days
4 Acetate buffer 70 gm of sodium *acetate* ($NaC_2H\,O,\ 3H_2O$) are dissolved in 100 gm of water and 15 ml glacial acetic acid added
5 45 per cent urea.
6 Winkler's solution 20 gm of cuprous chloride 25 gm of ammonium chloride and 75 gm of water are pla ed in a bottle just large enough to contain them *The bottle is corked, shaken with as little air as possible* and the precipitate then allowed to settle A coil of copper wire is placed in the solution which is then covered with a layer of heavy oil After some time the *reagent becomes almost* colorless.
7 Pyrogallol solution 15 gm of powdered pyrogallol are added to 100 ml of 0 *per cent* NaOH in a rubber stoppered bottle and covered with a layer of oil. The pyrogallol is dissolved under the oil by stirring with a glass rod.

The pyrogallol *solution is stored in a 10 ml syringe with the rear of the plunger* well greased and a fine tipped glass nozzle attached to the syringe The other reagents are similarly stored in smaller syringes The water is placed in a bottle above the apparatus and is delivered through rubber tubing with a *glass nozzle attached* A 20 ml syringe with a needle or glass nozzle attached can be used for the delivery of the water

Procedure

1 The syringe is held vertically and any liquid in the cup of the syringe is withdrawn by suction The plunger is pushed up and the cup filled with ferricyanide solution The solution is drawn down to the bottom of the syringe and expelled through the cup and removed. This procedure is repeated twice with *fresh lots* of ferricyanide the dead space finally being left full of ferricyanide without trapping any air bubbles No grease or oil is used in the syringe
2 The glass cup is filled to the mark with ferricyanide and the latter drawn down to the bottom of the cup
3 A drop of caprylic alcohol is deposited on the bottom of the cup
4. The pipette is filled with blood to the mark wiped and cautiously introduced into the glass cup and its *tip pressed snugly but not too vigorously against* the bottom of the cup The pipette should be held at a slight angle to the horizontal so that the blood does not run out when both ends of the pipette are open to the air
5 By pulling out the plunger gradually the blood is slowly and evenly drawn into the capillary followed by a bubble of air of about 1 mm length. If the tip is properly ground and the right amount of pressure applied no appreciable caprylic *alcohol is drawn in during this step The bubble of air prevents* any blood from being sucked back into the tip when the pipette is removed
6 The pipette is quickly removed and the bubble of air i then expelled thro gh the caprylic alcohol with the aid of the wire
7 A trace of caprylic alcohol i e about 2 divisions length of the capillary *is drawn* down onto the top of the blood and the rest of the caprylic alcohol is removed from the cup
8 The cup is filled to the mark with acetate buffer and the latter drawn down to the bottom of the cup
9 The cup is then immediately filled to the top with 45 per cent urea solution and then closed firmly with the finger
10 The arresting clip is then removed and the *closed apparatus* vigorously shaken in the horizontal position The plunger will be gradually drawn out by itself as the CO and other gases are evolved the gas pressure in the syringe being kept roughly atmospheric The total volume evolved is usually about 0·5 ml. Shaking is continued for a total of 2 minutes

11 The arresting clip is put back in place

12 The finger is cautiously released the gas meniscus being kept in the capillary by manipulating the plunger

13 A small amount of urea solution is allowed to run down into the capillary and left there until the walls are perfectly clean Then the rest of the urea solution (three quarters) is removed.

14 The rubber cup is adjusted and filled with pyrogallol solution Use the fine wire to avoid trapping of air bubble

15 A little of the pyrogallol solution is drawn into the syringe This absorbs some CO and O causing a partial vacuum which quickly sucks in more pyrogallol until only a small bubble consisting of N and CO (if any was originally present in the blood) is left at the top of the syringe The absorption takes a few seconds and just before it is complete the residual bubble is screwed slowly and carefully up into the capillary by manipulation of the plunger

16 The rubber cup is removed As it still contains a fair amount of pyrogallol it is advisable to remove cup under a stream of running water The glass cup is then emptied and filled again with water

17 The capillary is placed for half a minute in a beaker of water at room temperature and the temperature is read.

18 It is then removed dried by light wiping care being taken that the capillary is not handled and the volume of the bubble read V divisions

19 The glass cup is flushed clean with water and left filled. About three-fourths of this water is pulled quickly down into the syringe forming a layer on top of the heavier blood mixture The bubble with clean water below it is then at once run up to the top of the capillary

20 The glass cup is emptied of water and filled with Winkler s solution.

21 The syringe is then pointed with the cup downward the capillary making a slight angle with the horizontal

22 By cautiously screwing in the plunger the gas bubble is driven out into the glass cup where it rests near the junction of the capillary and the cup As soon as the bubble is free in the cup Winkler s solution is sucked behind it so as to half fill the capillary Gentle rotation for a few seconds completes the absorption of the CO

23 The syringe is then turned to the vertical position with the cup downward the gas bubble is sucked back into the capillary and its volume V measured.

Calculations

The carbon monoxide content of the blood in volumes per cent is

$$(V_1 - V_2) \times f \quad \text{where}$$

V_1 = First reading after absorption of the CO_2 and O_2.
V_2 = Second reading after absorption of the CO
f = Correction factor for temperature aqueous vapor pressure and barometric pressure as derived from Fig 23 at the beginning of the section on blood gases

Example

First reading V_1 = 5 5
Second reading V_2 = 4.2
Correction factor f = 0 900 at T = 2[illegible] C, bar press. = [illegible]60 mm.
ml CO/100 ml blood = (5.5 − 4.2) × 0 900 = 1.2

Precautions

1. When the blood is drawn from the pipette into the syringe care should be taken that the pipette is pressed snugly but not too vigorously against the bottom of the cup in order to prevent breakage of the tip.

3 Ferricyanide solution 12 5 gm of $K_3Fe(CN)_6$ 3 gm of $KHCO_3$ and 1.5 gm of saponin are ground in a mortar and dissolved in water and made up to 50 ml. The solution should be renewed every three days
4 Acetate buffer 70 gm of sodium acetate ($NaC_2H_3O_2$ $3H_2O$) are dissolved in 100 gm of water and 15 ml glacial acetic acid added
5 45 per cent urea.
6 Winkler s solution 20 gm of cuprous chloride 25 gm of ammonium chloride and 75 gm of water are placed in a bottle just large enough to contain them. The bottle is corked shaken with as little air as possible and the precipitate then allowed to settle A coil of copper wire is placed in the solution which is then covered with a layer of heavy oil After some time the reagent becomes almost colorless
7 Pyrogallol solution 15 gm of powdered pyrogallol are added to 100 ml of 0 per cent NaOH in a rubber stoppered bottle and covered with a layer of oil. The pyrogallol is dissolved under the oil by stirring with a glass rod.

The pyrogallol solution is stored in a 10 ml syringe with the rear of the plunger well greased and a fine tipped glass nozzle attached to the syringe The other reagents are similarly stored in smaller syringes The water is placed in a bottle above the apparatus and is delivered through rubber tubing with a glass nozzle attached A 20 ml syringe with a needle or glass nozzle attached can be used for the delivery of the water

Procedure

1 The syringe is held vertically and any liquid in the cup of the syringe is withdrawn by suction The plunger is pushed up and the cup filled with ferricyanide solution The solution is drawn down to the bottom of the syringe and expelled through the cup and removed This procedure is repeated twice with fresh lots of ferricyanide, the dead space finally being left full of ferricyanide without trapping any air bubbles No grease or oil is used in the syringe
2 The glass cup is filled to the mark with ferricyanide and the latter drawn down to the bottom of the cup
3 A drop of caprylic alcohol is deposited on the bottom of the cup
4 The pipette is filled with blood to the mark, wiped and cautiously introdu ed into the glass cup and its tip pressed snugly but not too vigorously against the bottom of the cup The pipette should be held at a slight angle to the h rizontal so that the blood does not run out when both ends of the pipette are open to the air
5 By pulling out the plunger gradually the blood is slowly and evenly drawn into the capillary followed by a bubble of air of about 1 mm length. If the tip is properly ground and the right amount of pressure applied no appreciable capry ic alcohol is drawn in during this step The bubble of air prevents any blood from being sucked back into the tip when the pipette is removed.
6 The pipette is quickly removed and the bubble of air is then expell d through the caprylic alcohol with the aid of the wire
7 A trace of capryli alcohol i e about ° divisions l ngth of the capillary is d awn down onto the top of the blood and the rest of the caprylic alcohol is removed from the cup
8 The cup is filled to the mark with acetate buffer and the latter drawn down to the bottom of the cup
9 The cup is then immediately filled to the top with 45 per cent urea solution and then closed firmly with the finger
10 The arresting clip is then removed and the closed apparatus vigorously shaken in the horizontal position The plunger will be gradually drawn out by itself as the CO and other gases are evolved the gas pressure in the syringe being kept roughly atmospheric The total volume evolved is usually about 0 75 ml. Shaking is continued for a total of 2 minutes

11 The arresting clip is put back in place.
1. The finger is cautiously released the gas meniscus being kept in the capillary by manipulating the plunger
13 A small amount of urea solution is allowed to run down into the capillary and left there until the walls are perfectly clean Then the rest of the urea solution (three quarters) is r moved.
14. The rubber cup is adjusted and filled with pyrogallol solution Use the fine wire to avoid trapping of air bubbles.
15 A little of the pyrogallol solution is drawn into the syringe This absorbs some CO and O causing a partial vacuum which qui kly sucks in more pyrogallol until only a small bubble consisting of N and CO (if any was originally present in the blood) is left at the top of the syring The absorption takes a few seconds and just before it is complete the residual bubble is screwed slowly and carefully up into the capillary by manipulation of the plunger
16 The rubber cup is removed. As it still contains a fair amount of pyrogallol it is a lvisable to remove cup under a stream of running water The glass cup is then emptied and filled again with water
17 The capillary is placed for half a minute in a beaker of water at room temperature and the temperature is read.
18 It is then removed dried by light wiping care being taken that the capillary is not handled and the volume of the bubble read V divisions
19 The glass cup is flushed clean with water and left filled About three fourths of this water is pulled quickly down into the syringe forming a layer on top of the heavier blood mixture The bubble with clean water below it is then at once run up to the top of the capillary
0 The glass cup is emptied of water and filled with Winkler s solution
21 The syringe is then pointed with the cup downward, the capillary making a slight angle with the horizontal.
2 By cautiously screwing in the plunger the gas bubble is driven out into the glass cup where it rests near the junction of the capillary and the cup As soon as the bubble is free in the cup Winkler s solution is sucked behind it so as to half fill the capillary Gentle rotation for a few seconds completes the absorption of the CO
"3 The syringe is then turned to the vertical position with the cup downward the gas bubble is sucked back into the capillary and its volume V *measured.*

Calculations

The carbon monoxide content of the blood in volumes per cent is

$$(V - V) \times f \quad \text{where}$$

V = F rst reading after absorption of the CO and O_2.
V = Second r ading after absorption of the CO
f = Correction factor for temperature aqueous vapor pressure and barometric pressure as derived from Fig .5 at the beginning of the section on blood gas s

Example

First reading V = 5.5
Second reading V = 4.
Correction factor f = 0 900 at T = C bar press = 760 mm
ml CO/100 ml blood = (.5 − 4.2) × 0 900 = 1.2

Precautions

1 When the blood is drawn from the pipette into the syringe care should be taken that the p pette is pressed snugly but not too vigorously against the bottom of the cup in order to prevent breakage of the tip

2 The arresting clip taken off at the beginning of the shaking should be put back in place before the releasing of the finger
3 Speedy adjustment of the bubble into the capillary at the end of the CO and O absorption with the pyrogallol helps to eliminate reabsorption of CO at the critical moment at which the partial pressure of the CO becomes high (i e nearly 1 atmosphere)
4 While there is gas in the syringe or bubbles in the capillary the hands should *not touch either one so as to prevent the effect of the hands' warmth on the* volume of the gases
5 Slipping back of the plunger is prevented by a proper adjustment of the arresting clip
6 Temperature equilibration of the bubble before the measurement of V has proved *necessary for with long bubbles a contraction of nearly a division may be ob*served as a result of immersion of the capillary in the water bath
7 To wash the instrument the plunger is pulled out under a stream of running water and the blood mixture poured out The syringe is filled and emptied several times with water before the plunger is restored. The plunger should never be *forced inward if there is a resistance due to precipitates inside it Occa*sionally the whole syringe should be rinsed with dichromate cleaning solution

B *SPECIAL METHOD FOR COMBINED ESTIMATION OF CO AND O IN SINGLE* SAMPLES OF BLOOD

Principle

When it is desired to measure both oxygen and carbon monoxide a single sample of blood can be used

Procedure

1 Follow Steps 1 through 20 in the procedure for oxygen and when V and V_1 are obtained
2 The glass cup and capillary are thoroughly washed with water no traces of the pyrogallol solution in the capillary in the cup being left
3 About three fourths of the clean water left in the cup is quickly pulled down into the syringe forming a layer on top of the heavier blood mixture The bubble with clean water below it is then at once run up to the top of the capillary
4 The glass cup is emptied of water and filled with Winkler s solution
5 The syringe is then held with the cup downward, the capillary making a slight angle with the horizontal
6 By cautiously screwing in the plunger the gas bubble is driven out into the glass cup where it rests near the junction of the capillary and the cup As soon as the bubble is free in the cup Winkler s solution is sucked behind it so as to half fill the capillary *Gentle rotation for a few seconds completes the absorp*tion of the CO
7 The syringe is then turned to the vertical position with the cup downward and the gas bubble is sucked back into the capillary and its volume V is measured.

Calculations

$(V_1 - V_2 - c) \times f =$ O content
$(V - V) \times f =$ CO content

Where

V = First reading after absorption *of the CO with NaOH*
V = Second reading after absorption of the O with pyrogallol
V = Third reading after absorption of the CO with Winkler s
f = Correction factor for temperature aqueous vapor pressure and *barometric* pressure as derived from Fig 25
c = Blank correction which in this case can be taken as 1 1

Example

First reading	=	6 8	(V)
Se ond reading	=	5 5	(V)
Third reading	=	4 0	(V)
Blank figure	=	1 1	(c)
Corr factor	=	0 900	(f) for Temp ° C bar press 760 mm

ml O_2 per 100 ml blood = (6 8 − 5 5 − 1 1) (0 900) = 18

ml CO per 100 ml blood = (5 5 − 4 0) (0 900) = 1 4

Precautions

All precautions given for the general method are applied here

C. SPECIAL METHOD FOR BLOOD CONTAINING SMALL AMOUNTS OF CARBON MONOXIDE

When the CO conte t of the blood is suspected to be small i e that it does not exceed °0 volumes percent this special method is used which requires three times (1 0 cmm) the amount of the blood sample used in the general m thod. Thus an accuracy of 0 03 to 0 05 volume percent is obtained.

Principle

The gases of the blood are extracted by means of the CO gas phase produced by adding an acetate buffer to a ferricyanide solution containing sodium bicarbonate and saponin in the u ual way described in the General Method. A special method is necessary because three times more blood is used than in the regular method and the relatively large amount of O l berated may cause an appreciable formation of CO during the pyrogallol absorption whereas when only 40 cmm are used as in the regular method no such CO formation has been detected To avoid th s extra formation of CO a weakly alkaline hydrosulfite is employed which will absorb the O of the blood.

Apparatus

The same apparatus described in the general method is used here However the pipette should have a mark where it has been calibrated to deliver thrice the usual blood volume

Reagents

Those reagents described under the general method for estimation of CO in blood are used together with the following

1 Hydrosulfite borate solution A stock sol ition of 3 per cent bo ax (Na_2B O 10H O) is prepared. 1 gm of sod um hydrosulfite (Na_2S_2O) is d ssolved in 50 ml of the borax solution and stored in a glass tipped syringe The final solut on should not be kept more than 2 days.

Procedure

1 The glass cup of the syringe is filled with hydrosulfite borate solut on which is drawn down to the bottom of the syringe a d then expelled leaving the dead space and the capillary filled with the solution

2 The blood is drawn down the capillary directly on top of the borate solution from a pipette calibrated to deliver 3 time the usual quantity of blood No caprylic alcohol is needed at this stage

3 The blood is drawn down to the bottom of the cap llary and the syringe rotated horizontally round its axis so as to m x the hydrosulfite with the blood

4 The syr nge is then restored to the vertical po t on and the blood solution brought to the top of the capillary

5 The cup is filled to the top with ferricyanide solut on and the whole of this drawn down

6 Two or more divisions of caprylic alcohol are placed on top of the ferricyanide solution in the capillary *so as to protect it from contact with the acetate buffer* used in the next step

7 The glass cup is rinsed with water and filled to the mark with acetate buffer

8 The acetate buffer is then drawn down a few divisions below the bottom of the cup The cup is then filled with water and closed at the top with the tip of the *finger as soon as the few divisions of air have been expelled from the top of the* capillary

9 The arresting clip is taken off and the gases are extracted in the usual way by 3 minutes shaking

10 The arresting clip is put back in place

11 The *syringe is then turned to the vertical position with the cup downward* and the plunger pushed in so as to give a slight positive pressure

12 With the syringe held over a dish the finger tip is removed gradually from the top of the cup and fluid is expelled until the meniscus is halfway down the capillary The gas containing part of the syringe chamber must not be touched *with the warm hand*

13 The glass cup is filled to the top with water and then slanted downward and the gas bubble pushed to the end of the capillary The blood mixture passes into the cup and settles through the water

14 With the instrument still slanting water is sucked into the capillary

15 The *instrument is then restored to the usual position (vertical)* with the cup upward and the latter filled with urea solution

16 The capillary is filled with urea solution and is left until the walls are *perfectly* clean

17 Three quarters of the urea in the glass cup is removed and the rubber cup adjusted and filled with pyrogallol solution without trapping air bubbles

18 A little pyrogallol is drawn down into the syringe This absorbs some CO and O causing a *partial vacuum which quickly sucks in more pyrogallol* until only a small bubble consisting of N and CO is left at the top of the syringe The absorption takes a few seconds and just before it is complete the *residual* bubble is screwed slowly and carefully up into the capillary by manipulation of the plunger

19 The rubber cup is removed As it still contains a fair amount of pyrogallol it is advisable to remove the cup under a stream of running water The glass cup is then emptied and refilled with water

20 The capillary is placed for half a minute in a beaker containing water at room *temperature and the temperature is read*

21 It is then removed dried by light wiping care being taken that the capillary is *not handled and the volume of the bubble read V divisions*

22 The glass cup is flushed clean with water and left filled. About three quarters of this water is pulled quickly down into the syringe *forming a layer on top of the* heavier blood mixture The bubble with clean water below it is then at once run up to the top of the capillary

23 The glass cup is emptied of water and filled with Winkler s solution

24 The syringe is then held with the cup downward the capillary making a *slight* angle with the horizontal

25 By cautiously screwing in the plunger the bubble is driven into the glass cup *where it rests near the junction of the capillary and the cup* As soon as the bubble is free in the cup Winkler s solution is sucked behind it so as to fill the capillary *Gentle rotation for a few seconds completes the absorption of the CO*

26 The syringe is then turned to the vertical position with the cup downward and the gas bubble is sucked *back into the capillary and its volume* V measured.

Calculations

$$\frac{(V - V) \times f}{3} = \text{CO content of blood}$$

Where

V = First reading after absorption of the CO and O

V = Second reading after absorption of the CO

f = Correction factor for temperature aqueous vapor pressure and barometric pressure as derived from Fig 25 at the beginning of the section on blood gases

(As thrice the usual amount of blood has been the usual result has to be divided by 3)

Example

First read ng 6 5 (V)
Second read ng 5 0 (V)
Corr factor 0 900 (f) When Temp - C bar press 760 mm

$$\frac{(6\,5 - 5\,0) \times 0\,900}{3} = 0\,45 \text{ ml CO per 100 ml blood}$$

Precautions

All those given for the general CO method are observed here

12 Carbon Dioxide in Blood (Scholander Roughton)

Reference

Scholander P F and Roughton F J W Micro Gasometric Estimation of the Blood Gases IV Carbon Dioxide J Biol. Chem. 148 573 580 (June) 1943

Principle

While carbon dioxide is by comparison ideal as an extractant for the microestima t on of the other gases and there is no practicable method using a gas other than CO for blood a modification of the technique was necessary in order to use the convenient syringe method for the estimation of CO This has been accomplished by the introduction of the vacuum extraction to the general method

The blood sample and an ac d buffer are vacuum extracted in the syringe by closing the capillary with the especially constructed rubber tipped plug and drawing the plunger out to the position fixed by a spacer (see Apparatus) and then shaking for 2 minutes The vacuum is released by letting in the plunger and the volume of gas is measured before and after absorpt on with alkali. The reabsorption of CO during the compress on is one of the special d fficulties but it is possible to check it by (a) using an acid buffer reagent of minimal CO solubility and (b) clearing the capillary of liquid before the shaking begins When the plunger moves up the whole of the gas therefore goes directly into the well drained capillary so that during the final stages of the contraction when the pressure of CO is approach ing atmospheric the gas is only in close contact w th the minute amount of liquid contained in the drainage films on the walls of the capillary

Apparatus

1 One ml Pyrex tuberculin syringe with arrest ng clip and with a standar l bore precision 0 mm Pyrex capillary fused to its nozzle The top of the capillary is expanded to a cylindrical cup of about 5 mm bore and 1 5 cm length The capillary (7 to 8 cm length) is graduated into 30 d vis ons each of - mm length An additional mark scratched at 33 3 has proved convenient when in an alte na tive procedu e it s de ed to use exactly one third of the normal p pette load

2 Blood pipette *made of thin walled glass tubing (1 to 1 5 mm bore)* with a ground tip to fit into the bottom of the glass cup The volume delivered from the mark *to the tip is equal to 100 divisions of the capillary* and is actually 39 3 cmm
3 Rubber tipped wooden plug to seal the capillary during the vacuum extraction. *The end of a round toothpick is dipped in rubber latex and a small drop left on* the tip It is dried tip downward at a moderate temperature in a drying oven. About 1 inch of the *coated plug is used as in Fig 35, A*
4 A spacer for holding out the syringe plunger in a fixed position during the vacuum extraction *A piece of light sheet metal about 1 5 cm wide* and 5.5 cm long is folded into a U shaped channel To apply the vacuum one end of the spacer is pressed against the expanded lower end (button) of the plunger and the latter is then drawn out until the other end of the spacer presses against the butt end of the syringe barrel The length of the spacer is so adjusted as to give a gas phase of about 0 75 ml in the barrel (See Figs 35A and B)
5 Several 2 ml and 5 ml syringes with fine tipped rubber connected glass nozzles to store the reagents
6 Metal or glass beakers one of them containing water at room temperature to hold the syringe for equilibration between readings
7 Suction line with a very fine tipped glass point
8 Thermometer for water bath

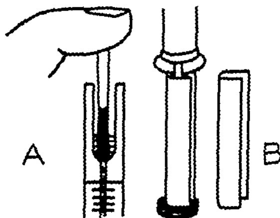


Fig 35—Carbon dioxide in blood
A Rubberized wooden plug for vacuum sealing glass cup
B Spacer for keeping plunger extended in fixed position during vacuum extraction.

Reagents

1 CO_2 free distilled water (Ordinary water can be freed of CO_2 by boiling with a drop of sulfuric acid.)
2 Caprylic alcohol.
3 Acid buffer 95 gm of acid sodium phosphate $NaH_2PO_4 \cdot H_2O$ are dissolved in 100 gm of warm water to make an almost saturated solution. The strength is about 5 M and the solubility of CO_2 therein is only about one twelfth that in water
4 10 percent NaOH
5 *Glycerol*

Reagents 2 3 and 4 may best be stored in 2 or 5 ml syringes with glass tips attached. The glycerol is best kept in a 2 ml syringe having a No 20 needle the point of which has been ground off The distilled water may be stored in a bottle with rubber tubing and glass tip attached hanging above the bench

Procedure

1 The clean syringe is taken apart and the water shaken out of the barrel and the plunger dried. The rear part of the plunger is lubricated with a few streaks of glycerol and returned to the moist barrel

2 The cup is filled with d stilled water and the latter is drawn one fourth down the barrel and expelled through the cup leaving the dead space of the syringe full of water without trapping any air bubbles

3 The plunger is pulled out very slightly so that the water meniscus at the bottom of the cup is lowered 1 or 2 mm down into the capillary

4. The blood pipette is held against the opening of the capillary trapping a small air bubble The blood is drawn down very slowly into the capillary the air bubble separating it from the water

5 If the blood supply is ample the quickest procedure is to dry the cup with a little cotton on a toothpick and then half fill it with blood trapping the small air bubble in the capillary The blood is then drawn down into the capillary the top layer being used as a protective against CO loss. If the blood is stored in a syringe it is convenient to attach a capillary glass t p to the nozzle and then afte good mixing and discarding of a few drops through the tip the cup is filled to the mark directly from the syringe tip

6 With the blood meniscus at the 30 to 35 mark the pipette is detached and the blood in the cup sucked off The upper blood meniscus is slowly moved to the zero mark and the amount of blood read b divisions

7 (As an alternative procedure the blood is moved down to a special mark scratched at 33 3 divis ons With a fine suction tip the upper meniscus is adjusted exactly to the zero mark the lower meniscus of the blood being simultaneously kept at the 33 3 mark In this way exactly one third of the normal pipette load is used and the volume of CO found has after the usual co rection for temperature etc simply to be multiplied by 3 to give the CO content in volumes percent)

8 A drop of caprylic alcohol is depos ted on the bottom of the cup and the bubble of air above the blood ejected through the capillary with the aid if necessary of a piece of fine wire. Two divisions of caprylic alcohol are drawn down onto the top of the blood in the capilla y The remainder of the capryl c alcohol in the cup is removed.

9 The cup is filled to the mark with acid phosphate which is then pulled down very slowly into the sy inge until the upper meniscus is mm below the bottom of the cup

10 The rubber end of the wooden plug is moistened with the phosphate buffer and with a few drops adhering to it is inserted in the bottom of the cup trapping a small air bubble

11 With the plug resting loosely against the bottom of the cup the air bubble is gently screwed up unt l it touches the rubber tip The plug is then pressed against the bottom of the cup leaving the air bubble in direct contact with the rubber The f ee lower end of the plug is covered by the drop of phosphate The capillary is kept closed in this way during steps (1") to (18) with the left hand

1 Fresh glycerol is introduced into the plunger bearing with the right hand

13 One end of the metal spacer is put in place around the plunger under the plunger head the right hand being used and is held there w th the other end sticking out at an angle The cup end of the syringe is pointed slant ng upward and kept upward during steps (14) to (17)

14 With the space and the plunger button held the plunger is slowly moved out in such a way that the fluid meniscus under the stopper moves down the capillary very slowly and evenly just as for a reading When the capillary and its barrel

opening are cleared and drained of the fluid, the plunger is drawn slowly out the free end of the spacer being simultaneously moved in until it rests against the butt of the syringe barrel

15 Glycerol is added to the plunger bearing

16 The syringe is shaken with the cup end upward so as to prevent any fluid from blocking the entrance to the capillary If nevertheless the capillary should become bridged over it can be cleared by warming up the capillary with the hand Shaking is continued for 2 minutes In case of foaming the plunger is released so that the fluid and extracted gas are compressed into the top part of the syringe barrel where there are normally residual traces of caprylic alcohol When the plunger is drawn out again the foam generally disappears

17 With the entire capillary free from fluid the plunger is pulled out slightly so as to allow the spacer to fall out The plunger is then allowed to rise up within the barrel at a controlled but rather rapid speed until the lower end of the meniscus is inside the capillary and the pressure of the gas is at atmospheric pressure If the entrance to the capillary should get bridged by fluid while the plunger is being let in the speed of the plunger must be so adjusted that the bridge moves very slowly up the capillary thus enabling proper drainage to take place

18 When the gas bubble is at atmospheric pressure the plug is removed and the upper meniscus moved slowly and evenly to the zero mark

19 The capillary is placed for ½ minute in a beaker of water at room temperature and the temperature is read It is then removed and dried by light wiping care being taken that the capillary is not handled

20 The volume of the bubble is read V divisions without any unnecessary adjustments as this may lead to appreciable reabsorption (the capillary should have been properly drained during step [14])

21 The cup is filled to the top with water and three fourths of the latter is pulled down into the syringe forming a layer on top of the blood mixture The bubble is returned to the capillary with water beneath it Owing to absorption of CO by this washing the bubble is much shorter

22 The cup is filled with 10 percent NaOH the cup pointed downward and the bubble expelled into the alkali some of which is drawn into the capillary as soon as the bubble is free

23 The cup is pointed slightly down and rotated a few times so as to complete the absorption of CO The syringe is then returned to the vertical position with the cup downward and then a gas bubble is sucked back into the capillary

24 *The temperature of the capillary is again adjusted and* the bubble volume read V

Calculations

The original formula to obtain the CO content of the blood by this method was as follows

$$\text{CO content} = (V \quad V) \times f \times \frac{100}{b}$$

Where

V = First reading

V = Second reading after absorption of the CO

f = Correction factor for temperature aqueous vapor pressure and barometric pressure as derived from Fig 25 at the beginning of the section on blood gases

b = Volume of blood expressed as the number of capillary divisions

No allowance was made however for the incomplete extraction of CO during shaking and for reabsorption during compression It was necessary therefore to run a series of determinations of CO by the syringe method and compare them with the results obtained by more precise a method like the Van Slyke By dividing the

opening are cleared and drained of the fluid the plunger is drawn slowly out, the free end of the spacer being simultaneously moved in until it rests against the butt of the syringe barrel

15 Glycerol is added to the plunger bearing

16 The syringe is shaken with the cup end upward so as to prevent any fluid from blocking the entrance to the capillary If nevertheless the capillary should become bridged over it can be cleared by warming up the capillary with the hand Shaking is continued for 2 minutes In case of foaming the plunger is released so that the fluid and extracted gas are compres ed into the top part of the syringe barrel where there are normally residual traces of caprylic alcohol When the plunger is drawn out again the foam generally disappears

17 With the entire capillary free from fluid the plunger is pulled out slightly so as to allow the spacer to fall out The plunger is th n allowed to rise up within the barrel at a controlled but rather rapid speed until the lower end of the meniscus is inside the capillary and the pressure of the gas is at atmospheric pressure If the entrance to the capillary should get bridged by fluid while the plunger is being let in the speed of the plunger must be so adjusted that the brid e moves very slowly up the capillary thus enabling proper drainage to take place

18 When the gas bubble is at atmospheric pressure the plug is removed and the upper meniscus moved slowly and evenly to the zero mark

19 The capillary is placed for ½ minute in a beaker of water at room temperature and the temperature is read. It is then removed and dried by light wiping care being taken that the capillary is not handled

20 The volume of the bubble is read V divisions without any unnecessary adjustments as this may lead to appreciable reabsorption (the capillary should have been properly drained during step [14])

21 The cup is filled to the top with water and three fourths of the latter is pulled down into the syringe forming a layer on top of the blood mixture The bubble is returned to the capillary with water beneath it Owing to absorption of CO by this washing the bubble is much shorter

22 The cup is filled with 10 percent NaOH, the cup pointed downward, and the bubble expelled into the alkali some of which is drawn into the capillary as soon as the bubble is free

23 The cup is pointed slightly down and rotated a few times so as to complete the absorption of CO The syringe is then returned to the vertical position with the cup downward and then a gas bubble is sucked back into the capillary

24 The temperature of the capillary is again adjusted and the bubble volume read V

Calculations

The original formula to obtain the CO_2 content of the blood by this method was as follows

$$CO \text{ content} = (V - V) \times f \times \frac{100}{b}$$

Where

V = First reading

V = Second reading after absorption of the CO

f = Correction factor for temperature aqueous vapor pressure and barometric pressure as derived from Fig 25 at the beginning of the section on b ood gases

b = Volume of blood expressed as the number of capillary divisions

No allowance was made however for the incomplete extraction of CO during shak ing and for reab orption during compression It was necessary therefore to run a series of determinations of CO by the syringe method and compare them with the results obtained by more pre is a method like the Van Slyke By dividing the

Reagents

1 Glycerol aquaresin mixture This mixture is made by mixing equal parts of the two reagents and storing it in a two ml syringe with a # 0 needle the tip of which has been ground off square (Aquaresin is obtainable from the Glyco Products Co Inc New York.)

2 Ferricyanide solution. Dissolve 1 gm of potassium ferricyanide and 0 5 gm of saponin in 10 ml of distilled water Then add 0 1 ml of the acetate buffer to the solution.

3 Caprylic alcohol Reagent grade

4 Acid phosphate buffer Dissolve 95 gm of acid sodium phosphate ($NaH_2PO_4 \cdot H_2O$) in 100 ml of distilled water This is almost a saturated solution

5 Sodium hydroxide a 10% solution in water

6 Distilled water

7 Pyrogallol solution Add 15 gm of powdered pyrogallol to 100 ml of 20% sodium hydroxide solution in a rubber stoppered bottle and cover the solution with a layer of oil 2 cm thick. The pyrogallol is dissolved by stirring it with a glass rod passing it through the oil

Reagents 2 and 7 are kept for current use in 2 or 5 ml syringes with glass tips attached through the rubber tubing

Procedure

1 Remove the plunger from the syringe rinse it well with hot water and dry it by suction This is easily done by attaching a small glass tube to the vacuum line and inserting this tube to the bottom of the barrel so that air is pulled through the warm capillary and barrel

2 Add a drop of glycerol aquaresin mixture into the barrel opening Lubricate the dry plunger with a few streaks of the glycerol solution and return it to the barrel adding enough of the solution so that no air is carried in with the plunger Unless the plunger is moved gently in the barrel the latter may break because of the viscosity of the glycerol solution When the plunger strikes the bottom the glycerol solution should rise only a few mm in the capillary

3 Fill the cup with the ferricyanide solution draw it down into the barrel and expel it without trapping any air bubbles Remove the excess ferricyanide leaving only the dead space filled with the ferricyanide solution

4 Place a drop of caprylic alcohol on top of the ferricyanide solution draw down 2 scale divisions of the alcohol and suck out the remainder Draw out the plunger until the fluid meniscus is lowered 1 to 2 mm into the capillary

5 Seat the plastic tipped wire plug in the cup trapping a small air bubble on top of the caprylic alcohol The rubber band must be strong enough to keep the capillary firmly closed by the plastic tip Add a little of the ferricyanide solution into the cup to complete the seal

6 Pull the plunger out slowly until the spring clip engages the notch in the plunger Then add glycerol aquaresin solution around the plunger bearing If the caprylic alcohol stays in the capillary it may be moved down the barrel by warming the capillary behind it in the hand Allow a few seconds for extraction and release the vacuum by letting the plunger in Expel the bubble and repeat the extraction.

7 Lower the meniscus of the extracted fluid 1 to 2 mm into the capillary Place the tip of the blood pipette firmly against the bottom of the cup trapping a small (0 5 to 1 division) air bubble between the blood and the extracted fluid Draw the blood slowly in until the length (A) of the air bubble can be measured. Continue to draw the blood in very slowly and evenly until 28 to 29 divisions have entered Remove the blood pipette suck off the excess blood adjust the up

Oxygen is released from chemical combination by ferricyanide and carbon dioxide is evolved by the addition of acid phosphate buffer The gases are vacuum extracted in the syringe selectively absorbed and measured in the capillary

Apparatus

1 The same syringe analyzer as used for the Scholander Roughton Blood Gas analysis method with modifications shown in Fig 36

2 The syringe analyzer described by Scholander and Roughton has been modified to make it easy to hold the plunger out during the vacuum extraction by providing a notch in the plunger which engages a spring steel clip To cut the notch draw the plunger out 0 8 ml and mark it 3 to 4 mm past the syringe barrel Cut the notch in the plunger with a triangular medium or fine India oil stone straight below the number of the plunger slanting the stone so that the spring clip will not disengage the notch during the vacuum extraction

PILLARY SYRINGE AND SLING FOR THE COMBINED CARBON DIOXIDE AND OXYGEN ME

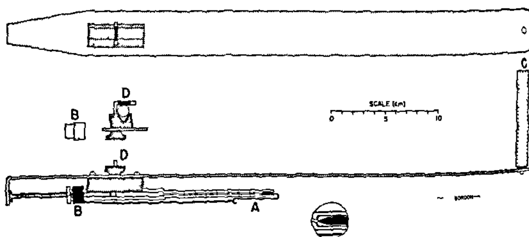


Fig 36

3 A plastic tipped wire plug to seal the capillary during the vacuum extraction. The tip is fashioned from a piece of 1 5 to 2 mm plastic cord by rotating and gently heating it in front of a micro flame and pulling it apart when the plastic becomes soft The pointed tip so formed is cut off to a length of 2 to 3 mm pierced straight with a fine wire which is bent A rubber band attaches the wire plug to the syringe barrel

4 A wire clip to give the plunger a suitable friction and to hold the plunger out during vacuum extraction A 6 to 7 mm wide ring is cut off from a piece of thick walled rubber tubing and is slipped firmly onto the syringe A straight angle bow of 0 4 to 0 5 mm of music wire wide enough to sl p over the syringe collar is stuck straight through the rubber tubing With the clip engaged in the plunger notch the end of the plunger should read 80 (0 8 ml)

5 A hand sling for the syringe for whirling down liquid bridges in the cap llary and for breaking up bubbles in the syringe The sling is 4 cm wide by 50 cm long made from 1 5 mm sheet aluminum It can be whirled around a handle The other end is bent out at a straight angle It is furnished with a plastic cradle which carries a screw hook padded with rubber tubing for fastening the syringe

Reagents

1 Glycerol aquaresin mixture This mixture is made by mixing equal parts of the two reagents and storing it in a two ml syringe with a # 0 needle the tip of which has been ground off square (Aquare n is obtainable from the Glyco Products Co Inc. New York)

2 Ferricyanide solution Dissolve 1 gm of potassium ferricyanide and 0 5 gm of saponin in 10 ml of distilled water Then add 0 1 ml of the acetate buffer to the solution

3 Caprylic alcohol Reagent grade

4. Acid pho phate buffer D ssolve 95 gm of acid sodium phosphate ($NaH_2PO_4 \cdot H_2O$) in 100 ml of distilled water This is almost a saturated solution

5 Sodium hydroxide a 10% solution in water

6 Distilled water

Pyrogallol solution Add 15 gm of powdered pyrogallol to 100 ml of 20% sodium hydroxide solution in a rubber stoppered bottle and cover the solution with a layer of oil cm thick The pyrogallol is di solved by stirring it with a glass rod passing it through the oil.

Reagents ° and are kept for current use in 2 or 5 ml syringes with glass tips attached through the rubber tubing

Procedure

1 Remove the plunger from the syringe rinse it well with hot water and dry it by suction This is easily done by attaching a small glass tube to the vacuum line and inserting th s tube to the bottom of the barrel so that air is pulled through the warm cap llary and ba rel

2 Add a drop of glycerol aquaresin m xture into the barrel opening Lubricate the dry plunger with a few streaks of the glycerol solution and return it to the barrel add ng enough of the solution so that no air is carried in with the plunger Unless the plunger is moved gently in the barrel the latter may break because of the viscosity of the glycerol solution When the plunger strikes the bottom the glycerol solution should rise only a few mm in the capillary

3 Fill the cup w th the ferricyanide solution draw it down into the barrel and expel it without trapping any air bubbles. Remove the excess ferricyanide leaving only the dead space filled with the ferricyanide solution.

4 Place a drop of capryl c alcohol on top of the ferricyanide solution draw down scale d visions of the alcohol and suck out the rema nder Draw out the plunger until the flu d meniscus is lowered 1 to 2 mm nto the cap llary

5 Seat the plastic tipped wire plug in the cup trapping a small air bubble on top of the cap ylic alcohol The rubber band must be strong enough to keep the capillary firmly closed by the plastic t p Add a l ttle of the ferricyanide solu tion into the cup to complete the seal.

6 Pull the plunger out slowly until the spr ng clip engages the notch in the plunger Then add glycerol aquares n solution around the plunger bearing If the caprylic alcohol stays in the capillary it may be moved down the ba rel by warming the capillary behind it in the hand Allow a few seconds for extraction and release the vacuum by letting the plunger in. Expel the bubble and repeat the ex tract on

7 Lower the meniscus of the extracted fluid 1 to 2 mm into the capillary Place the t p of the blood pipette firmly aga nst the bottom of the cup trapping a small (0 5 to 1 division) air bubble between the blood and the extracted fluid. Draw the blood slowly in unt l the length (A) of the air bubble can be measured. Continue to draw the blood in very slowly and evenly until 28 to 29 divisions have ente ed Remove the blood pipette suck off the exces blood adjust the up-

per blood meniscus to the zero mark, and read the volume of the blood sample b divisions (With practice the blood volume can be made *very close to 30* divisions)

8 Fill the cup to the mark with the acid phosphate solution, leaving a small (0.5 *to 1 division*) air bubble between the buffer and the blood. Draw it slowly in until the length (A_1) of the air bubble can be measured. Turn the syringe cup down and continue to draw in the phosphate buffer slowly and evenly until the bubble reaches the junction of the capillary with the syringe barrel, preventing the acid from entering the syringe barrel

9 *Rotate the syringe on an oblique axis with the cup down until the blood is* completely laked to a uniform dark brown color by the ferricyanide saponin solution If more than a trace of acid buffer enters the blood will not lake properly and the oxygen will not be completely released from the hemoglobin.

10 Hold the syringe cup up Draw in the remainder of the acid phosphate solution and close the capillary with the plastic tipped wire plug trapping a small air bubble (0 5 division or less) underneath Estimate the length of this bubble (A_2) Then add a drop of acid buffer to the cup to complete the vacuum seal.

11 Pull the plunger out slowly so that the capillary drains properly when the small bubble underneath the plug expands Continue until the plunger notch engages the clip Again add the glycerol solution around the plunger bearing

12 Attach the syringe to the sling It is necessary that the plunger head should rest against the bent end of the sling Whirl the blood solution down to the *bottom of the syringe and tilt back and forth several times to* have the liquids well mixed Whirl the sling again for about 30 seconds The capillary should be clear of bridges and the blood solution free of bubbles

13 Remove the syringe from the sling without touching the capillary, and hold the cup up Release the plunger stop and move the fluid quickly up into the capillary until the atmospheric pressure is almost reached

14. Without delay remove the plug and adjust the upper gas meniscus to the zero mark and read the volume (V_1) of the gas bubble Since the capillary has not *been handled at any time there is no necessity for temperature equilibration* in a water bath (Delay and fumbling in Steps 13 and 14 result in some reabsorption of CO_2)

15 Fill the cup with water and with the syringe held cup up, pull most of the water down into the syringe where it will form a layer on top of the heavy blood solution Immediately return the bubble to the capillary leaving water behind it on both sides of the gas in the capillary Owing to the absorption of CO by this washing the bubble is now much shorter

16 Remove the excess water and *fill the cup with NaOH solution. Invert* the syringe run the gas bubble out into the alkali, and rotate a few times to facilitate absorption Draw some alkali into the capillary and then the gas bubble Measure its volume (V)

17 Remove the NaOH solution from the cup and fill it with pyrogallol solution. Run the bubble *out* into the cup and absorb as in the previous step Return the bubble to the capillary and read the volume record and designate it V_2 in calculations

18 Remove the plunger *gently from the barrel under the water faucet* Shake out the contents of the barrel and wash with running tap water several times before the plunger is again inserted. It is recommended that dichromate cleaning solution be used after each analysis to insure that no protein remains sticking to the capillary While the syringe is still hot dry it by suction as described in Step 1

Calculations

Carbon dioxide

Volumes percent of CO in blood = (V - V - c) × f × 100/b × i.

1 c_1 is the blank correction for the CO content of the reagents (It is determined by running an analysis with all the reagents but no blood It is usually about 0 1 capillary divisions)

f is the factor from Fig 5 for adjusting gas volumes from the observed temperature barometric pressure and water vapor to their dry volumes at 0 and 760 mm

3 b is the blood volume in terms of division of the capillary

4 i is the combined correction factor for the incomplete extraction of CO and for the CO reabsorbed by the solutions It was found to average 1 015 which s the same figure as that determined in the syringe method for CO in blood

Oxygen

Volumes per cent of O in blood = (V - V - c - a) × f × 100/b × i.

1 c is the blank correct on for the O contents of the reagents used. (Usually about 0 capillary division)

2 a is the O content of the air bubbles introduced during analysis in order to separate reagents a = 21 per cent (A + A + A)

3 f is the factor for the adjustment of moist gas at observed temperature and barometr c pressure to the dry condition at 0 and 760 mm (see Fig 5)

4 i is the combined correction factor for the incomplete extraction of O and for the reabsorption of O by the solutions It was found to average 1 011 (The factor was obtained as the average of the fa tors by which the results w th the syringe [in other respects corrected] must be multiplied to equal the results of the Van Slyke analys s)

14 Manometric Methods Miscellaneous References

Amino Nitrogen

Chinard F P and Van Slyke D D Compa i on of a Modified Folin Photometric Pro e dure and the N nhydrin Manometric Method fo the Determination of Amino Acid Nitrogen n Plasma J Biol Ch m 169 (3) 5 1 581 (Aug) 194

Van Slyke D D Improved Methods in the Gasometr c Determination of Free and Conjugated Amino Acid Nitrogen in the Urine J Biol. Chem 16 1 5 134 1913

Van Slyke D D Manometric Determination of Pr mary Amino Nitrogen and Its Applicat on to Blood Analysis J Biol Chem 83 4 5 447 (May) 19 9

Van Slyke D D MacFadyen D A and Hamilton P B The Gasometric Determination of Amino Acids n Urine by the Ninhyd n Ca bon Dioxide Method J B ol Chem 150 51 58 (Sept) 1943

Bicarbonate

Van Slyke D D and Cullen G E Stu lies of Ac do I The B ca b ate Co centra tion of the Blood Plasma Its S gn f cance and It Dete m n t o a a Measure of Ac dosis J Biol Chem 30 89 340 (May) 191

Carbon Dioxide

Van Slyke D D Sendroy J Jr and Liu S H Manometr An lys of Gas Mixtures III M nomet c Determ nat on of CO Ten ion and pH of Blood J Biol Chem 95 4 508 (March) 19

Carbon Monoxide

Van Slyke D D and Rob chert Robbins F S The Gasometric Dete mination of Small Amounts of Carbon Monoxide in Blood and Its Application to Blood Volume Stud es J Biol Chem 72 39 0 (March) 19 7

Hemoglobin

Van Slyke D D and Hiller A Gasometr c Determinat on of Hemoglobin by the Carbon Monoxide Capac ty Method J Biol Chem 78 807 8 0 (Aug) 19 8

Hydrogen

Van Slyke D D, and Hanke M E *Manometric Analyses of Gas Mixtures IV* Manometric Determination of Hydrogen and Oxygen by Combustion, J Biol Chem 95 569 586 (March) 1932

Van Slyke D D and Hanke M E Manometric Analysis of Gas Mixtures V Manometric Analysis of Gas Mixtures Hydrogen Absorption With Paal s Picrate Palladium Solution J Biol Chem 95 587 598 (March) 1932

Iodate

Van Slyke D D Hiller A and Berthelsen K C A Gasometric Micromethod for Determination of Iodates and Sulphates and Its Application to the Estimation of Total Base in Blood Serum J Biol Chem 74 659 676 (Sept) 1927

Lactic Acid

Avery B F and Hastings A B *A Gasometric Method for the Determination of Lactic Acid in Blood* J Biol Chem 94 273 280 (Nov) 1931

Methemoglobin

Van Slyke D D and Hiller A Gasometric Determination of Methemoglobin J Biol. Chem 84 205 216 (Oct) 1929

Nonprotein Nitrogen

Stehle, R L Gasometric Determination of Nitrogen and Its Application to the Estimation of the Nonprotein Nitrogen in the Blood J Biol Chem 45 223 228 (Dec) 19 0

Oxalic Acid

Van Slyke D D and Sendroy J Jr Gasometric Determination of Oxalic Acid and Calcium and Its Application to Serum Analyses J Biol Chem 84 217 3 (Oct) 1929

Potassium

Kramer B and Gittleman I The Gasometric Determination of Potassium Proc Soc Exper Biol & Med. 24 241 243 (Oct June) 1926 1927

Sugar

Van Slyke D D and Hawkins J A A Gasometric Method for the Determination of Reducing Sugars and Its Application to the Analysis of Blood and Urine J Biol Chem 79 739 768 (Jan) 1927

Van Slyke D D and Hawkins J A Gasometric Determination of Fermentable Sugar in Blood and Urine J Biol Chem 83 51 70 (May) 19 9

Total Nitrogen

Van Slyke D D Gasometric Micro Kjeldahl Determination of Nitrogen J Biol Chem 71 235 248 *(Dec) 19 6*

Van Slyke D D and Kugel V H Improvements in Manometric Micro Kjeldahl and Blood Urea Methods J Biol Chem 102 489 498 (Oct) 1933

Urea

Mirkin A A Gasometric Method for the Determination of Urea Nitrogen in the Blood J Lab & Clin Med. 8 (1) 50 52 (Oct) 1922

Van Slyke D D Determination of Urea by Gasometric Measurement of the Carbon Dioxide Formed by the Action of Urease J Biol Chem 73 695 724 (June) 192

SECTION VI

PHYSIOLOGICAL MEASUREMENTS (Continued)

B PHYSICAL CHEMISTRY OF BLOOD GASES

1 Technique for Equilibrating Arterial and Venous Blood Against Gas Mixtures of Known Composition

Reference

Henderson, L. J Blood New Haven Conn 19?8, Yale University Press Appendix page 388 by Dill D B

Principle

Blood is equilibrated at known tensions of carbon dioxide and oxygen in a constant temperature bath for a known period of time The blood is then analyzed in the Van Slyke apparatus and the gas phase is analyzed in the Haldane gas analysis apparatus This technique was used extensively by Henderson Bock Dill Edwards Talbott and associates in their studies of blood as a physicochemical system

Apparatus

1 Tonometer Barcroft type (see Fig 37)
? A number of syringes 5 and 10 ml
3 A burette 100 ml (see Fig 37)
4 A number of 50 ml round bottom centrifuge tubes
5 A number of # 0 gauge needles (sterilized)
6 A water bath constant temperature set at 37 C This bath contains a device for rotating blood tonometers
7 A number of blood sampling tubes for the storage of the blood
8 A Haldane gas analyses apparatus as in Fig 41
9 A Van Slyke manometric apparatus as in Fig 8
10 A rubber bag for the storage of gases
11 A number of glass tubes with capillary bore of ? mm

Reagents

1 A tank of oxygen
2 A tank of carbon dioxide
3 A tank of nitrogen
4 Clean mercury
5 Stopcock grease (Lubriseal A H Thomas Co)
6 Heparin solution One drop should inhibit coagulation of 10 ml blood for ?4 hours

Procedure—Preparation of Gas Mixture

1 The tonometer in which the blood is to be equilibrated is ordinarily prepared in advance of the blood drawing These tonometers are of the Barcroft type (see Fig 37) and have a capacity between ?80 to 3 0 ml The capacity is etched on each and serves as an identifying mark. The dry tonometer with the stopcock well lubricated and held in by rubber bands, is fitted with a rubber stopper

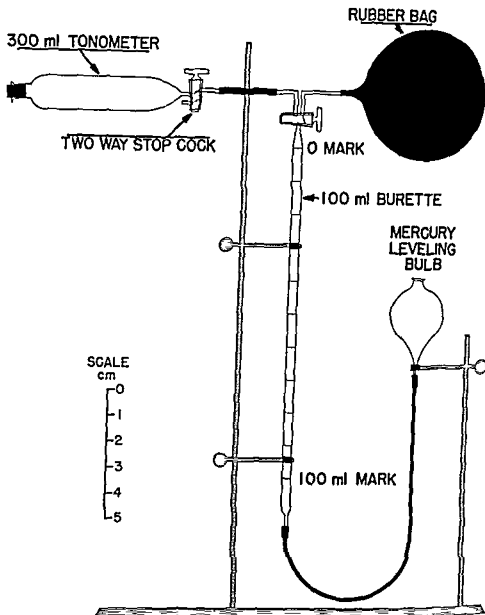


Fig 37 —Apparatus u ed by Henderson and associates for equilibrating blood with gas mixtures of known composition

Since in the calculation of arterial pH we are primarily interested in a partial pressure of 40 mm CO we shall describe specifically the preparation of a mixture with pCO 40 mm Hg and pO 180 to 00 mm H_g The same general technique is used for preparing tonometers for any other partial pressures.

- The general formula for computing the amount of gas to be added is

ml gas to be added to tonometer =

$$\frac{(\text{p Gas}) \times (\text{Volume of tonometer})}{(\text{barometric pressure})} \qquad \text{(Equation 1)}$$

For example pCO wanted is 40 mm pO wanted is 200 mm capacity of tonometer is 300 ml and barometric pressure is 760 mm Hg

Therefore $\text{ml CO} = \frac{40 \times 300}{60} = 15$ 8 ml and

$\text{ml O} = \frac{00 \times 300}{60} = 9\,0$ and

Sum of CO O = (15 8 9 0) = 94 8

3 Filling the tonometer is done as follows

a. The tonometer is first flushed out with nitrogen and then connected to the 100 ml burette

b With the tonometer stopcock open to the outside air and with stopcock of the burette open to the tonometer stopcock the gas burette is filled with mercury by raising the mercury leveling bulb

c The tonometer stopcock is turned and 94 8 ml of gas is removed from the tonometer

d. The tonometer stopcock is again turned and the gas is discharged through the side outlet

e The burette stopcock is turned to the rubber bag containing oxygen and the burette connections are flushed out with the oxygen twice

f Then measure out accurately 79 0 ml of oxygen in the burette and deliver it into the tonometer

g The bag of oxygen is replaced with a bag of carbon dioxide and after flushing out the bu ette and connect ons through the side outlet 15 8 ml of carbon di oxide are measured into the burette and then introduced into the tonometer

h When more than 15 ml of gas is removed from the tonometer account should be taken of the lowered pressure This may be done approximately by suitable lowering of the leveling bulb or the volume of gas withdrawn may be measured by closing the burette stopcock.

Procedure Drawing Preparation and Analysis of Arterial Blood

1 For the collection of arterial blood round bottomed centrifuge tubes of 50 ml capacity are used In the bottom are placed 4 drops of heparin solution and paraffin o l up to a he ght of 10 cm

- After establ shment of local anesthesia with one per cent Novocain blood is withdrawn from the radial or brachial artery

3 Particular care is taken in the case of arterial blood to avoid contact with the air The syringe is turned up the needle replaced with a glass capillary tube 2 × 100 mm and blood is pushed through to the tip of the tube The syringe is inverted and with the tube leading into the oil in the 50 ml centrifuge tube the blood is rapidly transferred It is then stirred carefully until the heparin is in solution with the blood (Any specimen of arterial blood normally represents the blood leaving the lungs in respect to its oxygen and carbon dioxide contents whereas arm vein blood may differ greatly from vena cava blood. Consequently it is of great interest to know the exact carbon dioxide and oxygen content of

arterial blood and of but little interest to secure such information with regard to a sample of venous blood) At this point one man proceeds promptly to the determination of oxygen and carbon dioxide contents of the arterial blood by the Van Slyke techniques

Procedure—Equilibration of Arterial Blood

1 With a 5 ml syringe 5 ml of arterial blood is introduced into the tonometer through its stopcock

2 With another syringe mercury is forced through the stopcock until the capillary bores are exactly filled

3 The time is noted and the tonometer is placed in a constant temperature bath set at 37 C where it is held in a mechanical rotator

4 Twenty minutes equilibration insures practically complete equilibrium under the above defined conditions

5 At the end of 20 minutes, the tonometer is clamped in an upright position in the water bath and a blood sampling tube is attached with a short piece of small bore tubing The sampling tube is like the gas sampling tube except that the capacity is only 6 ml

6 The tonometer stopcock is turned to the side arm the sampling tube stopcock is opened and by raising the leveling bulb some mercury is forced through the connection

7 The tonometer stopcock is then turned and by lowering of the leveling bulb the blood is drawn into the sampling tube

8 After closing of both stopcocks the rubber connection is removed and sampling tube and tonometer are taken from the bath

9 The sampling tube is placed in a rack and sealed off with mercury

10 The sample is kept at 5 C until it is analyzed for oxygen and carbon dioxide This is done after mixing the blood in the sampling tube thoroughly by inversion. The stopcock is carefully opened until all the mercury is expelled from the capillary bore A number 0 rubber stopper with a small bore makes a satisfactory connection with a Van Slyke Neill pipette The leveling bulb is supported in the rack slightly above the level of the sampling tube stopcock so that there is a positive pressure to fill the pipette when the stopcock is opened After the blood sample has be n introduced into the Van Slyke apparatus the sampling tube is again sealed off with mercury

Procedure—Analysis of the Gas Phase

1 *In general* the partial pressure of gas in the tonometer at 37 C is calculated from the gas pressure and the temperature of the tonometer at the time *of analysis*, according to the following equation

pGas at 37 C =

$$\frac{\%\ \text{Gas}}{100} \times (B - pH_2O + p) \times \frac{(310)}{(273 + T)} \times \frac{(V + H - 1)}{(V - b)} \qquad \text{(Equation)}$$

Where % Gas is percentage as analyzed B is barometric pressure mm Hg pH_2O is partial pressure of water at temperature of analysis p is the difference in pressure (tonometer minus barometer) at time of analysis V is volume *of* tonometer H is the volume of gas drawn into the Haldane apparatus for *analysis* and b is the volume of blood during equilibration One ml of blood always *stays* on the walls of the tonometer

2 In analyzing the gas phase the steps are as *follows*

a Dry the tonometer and allow it to come to room *temperature* *Expel* blood from the exterior ends of the tonometer cock

b Connect to the Haldane apparatus

c Expel residual gas from the Haldane apparatus

d. Connect to tonometer and use Haldane leveling bulb to draw gently the residual drop of blood from the interior bore of the tonometer cock and to expel it into the side bore of the exterior cock

e Draw 7 ml of gas into the Haldane

f Read difference in mercury levels to the nearest mm this figure p is used in the calculation

g Continue to flush out the dead space and analyze for CO and O Note that for fully oxygenated blood the pO is always very high and the value can be assumed without serious error

Example of Calculation

1 % CO was 5 00 B was 760 mm T at analysis was 5 C p which is tonometer minus barometer was minus 6 mm Volume of gas drawn into Haldane was 7 ml Tonometer 300 ml blood ɔ ml pH O at ɔ C is °4 mm Hg

Therefore pCO mm Hg =

$$\frac{5}{100} \times (760 - {}^{o}4 - 6) \times \frac{310}{\text{ɔ}73 + \text{ɔ}} \times \frac{(300 + 7 - 1)}{(300 - \text{ɔ})} = 40\ 0$$

° The values at different temperatures for pH O and the factor 310/(ɔ73 + T) are consolidated into Table 18

TABLE 18

PARTIAL PRESSURE OF WATER AND TEMPERATURE FACTOR FOR EQUILIBRATED BLOOD AT DIFFERENT ROOM TEMPERATURES

ROOM TEMPERATURE C	pH O mm Hg	FACTOR 310/(°73 + T) f
0	17	1 0ɔ8
21	19	1 054
	0	1 051
23	°1	1 047
°4	°ɔ	1 044
5	°4	1 040
26	5	1 037
27	ɔ7	1 033
ɔ8	8	1 030

2 Dissociation Curves of Blood

References

1 Henderson L. J Blood New Haven Conn 19 8 Yale University Press

° Dill, D B Graybiel A Hurtado A. and Taquini A. C Der Gasaustausch in den Lungen im Alter Sond aus Zeits Alters II (1) 0 33 1910

Calculations Involving Oxygen and Hemoglobin

1 Total Oxygen of Blood. Total oxygen of blood (total O)ₙ is calculated from results of analysis according to the method of Van Slyke and Neill (19 4) as described previously

2 Physically Dissolved Oxygen of Blood (O) According to the solubility studies of Send oy D llon and Van Slyke (1934) one liter of normal blood will dissolve 0 00138 millimole (mM) of O at 37 C and at 1/760 atmosphere Hence

(O)ₙ in mM/l = (0 00138) × (pO) or (Equation 1)

(O) in ml O_2/100 ml = (0 0031 × (pO) (Equation ɔ)

3 Oxygen Bound by Hemoglobin This is calculated as follows

(HbO) = (total O)ₙ − (O) (Equation 3)

4 Oxygen Capacity This figure (HbO capacity)$_b$ is (HbO)$_b$ of blood saturated with oxygen at pO 180 to 200 mm Hg at 37 C

5 Percentage Oxygen Saturation

$$\% \text{ Saturation} = \frac{100 \times (\text{HbO})_b}{(\text{HbO}_2 \text{ capacity})_b} \qquad \text{(Equation 4)}$$

6 Hemoglobin in Blood. The hemoglobin is calculated from the oxygen capacity as follows

$$\text{gm Hb/100 ml blood} = \frac{\text{Oxygen capacity ml O /100 ml blood}}{1\,34} \qquad \textit{(Equation 5)}$$

Calculations Involving Carbon Dioxide

1 Total CO of Blood This value (total CO)$_b$ is calculated after Van Slyke and Sendroy (1927)

2 Glycolysis During Equilibration Bock and associates (19?9) calculated that (total CO_2) is reduced about 0 4 vols % in 15 mins at 37 C This quantity is added to (total CO)$_b$ of equilibrated blood

3 Carbon Dioxide Content of Blood Equilibrated at 37 C and pCO 40 Corrected for Glycolysis This value T_{40} may be found from the curve of Henderson (1930) in Fig 67 at the back of this book The data required are (HbO capacity)$_b$ and (total CO_2)$_b$ at known pCO From these data (total CO_2)$_b$ at any other pCO may be derived Corrected for glycolysis this value at pCO 40 is called T_{40}

4 *Total Carbon Dioxide in Plasma If the equilibrated blood is handled under* anaerobic conditions direct analysis of the plasma by the technique of Van Slyke and Neill gives the total carbon dioxide (total CO_2) As an alternative, Henderson (1928) developed Fig 38 for computing Δ (CO) difference between (total CO_2) and (total CO_2) when T_{40} and (HbO capacity)$_b$ are known

$$(\text{Total CO}_2) = (\text{total CO})_b + \Delta(\text{CO}) \qquad \text{(Equation 6)}$$

5 *Free Carbonic Acid of Serum (H CO) From solubility coefficients of* Van Slyke (19?8) one liter of normal serum will dissolve 0 0313 mM of CO at 37 C and at 1/760 atmosphere Hence

(H_2CO) *in mM/l = (0 0313) × (pCO)* *(Equation 7) or*

(H CO) in ml CO /100 ml = (0 0696) × (pCO) (Equation 8)

6 Bicarbonate in Serum (BHCO) This value is computed from the equation

(BHCO) in mM/l = (total CO) − (H CO) *(Equation 9)*

7 Serum pH This value, pH is calculated from the Henderson Hasselbalch equation

pH = pK + log (BHCO) − log (H CO) (Equation 10)

Dill Daly and Forbes (1937) determined that the pK of normal human serum at 37 C is 6 11

Calculation of Arterial pCO

1 *Knowing* CO *combining capacity* (T_{40}) and the CO content of arterial blood *as drawn* and the oxygen combining capacity one calculates pCO_2 of the blood *as drawn from* Fig 67 at the back of the book as follows

a Place string at T_{40} on the left and 40 mm on P scale at right

b Fix a pin on A scale corresponding to the oxygen capacity in *ml/100 ml* blood

c Now swing the string about the pin setting the the *left hand figure on the* T scale at the CO content as analyzed.

d Keeping string straight read pCO on right hand scale

2 *Under ordinary conditions* the CO combining capacity *of arterial blood may* be taken as 0 1 mM greater than that of oxygenated *blood. Under unusual* conditions the correction must be arrived at more *precisely After correction*

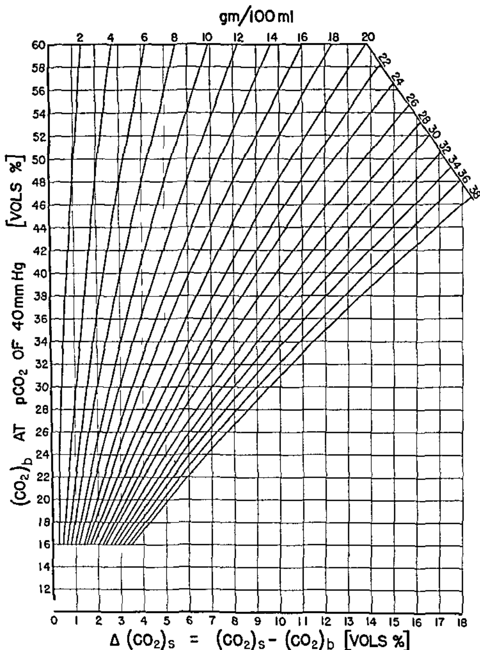


Fig 38 —H nd son s chart f r computi g whole blood and se um a bon di xide for blo d with different c n nt ation of hemogl bin.

is made for dissolved oxygen the proportion of reduced Hb is calculated The CO_2 combining capacity of arterial blood then is

CO_2 combining capacity =

$$T_{40} + \frac{\Delta\ (CO_2)_b \times (Hb)}{(HbO_2\ capacity)_b} \qquad \text{(Equation 11)}$$

Where T_{40}, $(Hb)_b$ and $(HbO_2\ capacity)_b$ are as defined above and $\Delta\ (CO_2)_b$ is the reduction of CO_2 combining capacity for increasing concentrations of oxyhemoglobin To determine the value of $\Delta\ (CO_2)_b$ from the T_{40} of oxygenated blood and (HbO_2 capacity) use Fig 39

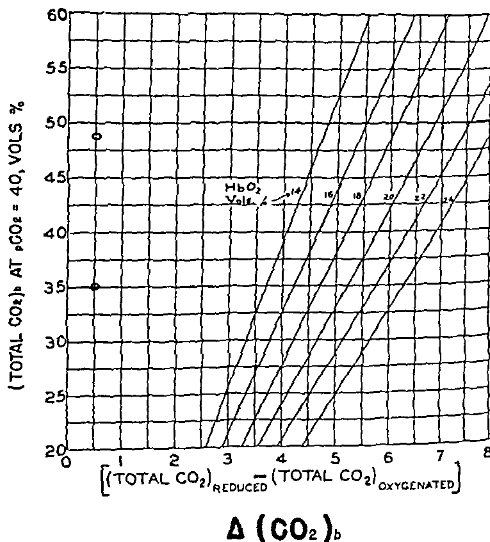
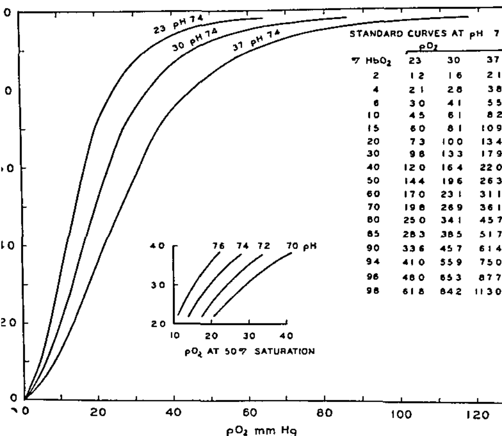


Fig 39 —Henderson's chart for determining the effect of oxygenation on the T_{40} of whole blood.

Dissociation Curves

1 For both oxygen and carbon dioxide, dissociation curves are plotted with some function of gas content as ordinate against some function of gas pressure as abscissa.



STANDARD CURVES AT pH 7

% HbO_2	pO_2 23	30	37
2	1 2	1 6	2 1
4	2 1	2 8	3 8
6	3 0	4 1	5 5
10	4 5	6 1	8 2
15	6 0	8 1	10 9
20	7 3	10 0	13 4
30	9 8	13 3	17 9
40	12 0	16 4	22 0
50	14 4	19 6	26 3
60	17 0	23 1	31 1
70	19 8	26 9	36 1
80	25 0	34 1	45 7
85	28 3	38 5	51 7
90	33 6	45 7	61 4
94	41 0	55 9	75 0
96	48 0	65 3	87 7
98	61 8	84 2	113 0

OXYGEN DISSOCIATION CURVES, HUMAN BLOOD, AT DIFFERENT TEMPERATURES

Fig 40—Effects of temp atu e and pH on xygen di ciation curves (acco ding to Dill and as ociates)

2 Points on the oxygen dissociation curves are obtained by preparing gas mixtures of suitable composition followed by precise determination of both pCO_2 and pO_2 in the gas phase The oxygen dissociation curve is usually plotted to show % HbO_2 as a function of pO_2 the pCO being constant. An example of this kind of curve is shown in Fig 40

3 The logarithmic oxygen dissociation curve plots log $\frac{100(Hb)_b}{(HbO)_b}$ against log pO_2, where $(Hb)_b/(HbO)_b$ is the ratio of reduced to oxygenated hemoglobin This curve has two advantages over the usual oxygen curve It is nearly straight and the effect of a given change in pH (or in temperature) on the log pO is the same for all values of 100 (Hb)/(HbO)

3 Miscellaneous Calculations of the Physicochemical Properties of Blood

Calculation of the Donnan Ratio

Reference

Henderson L. J Blood New Haven Conn 1928 Yale University Press

Calculation

1 As applied to blood the Donnan ratio is the ratio of the concentration of an ion in the cells divided by its concentration in the plasma concentration being best expressed as mEq of electrolyte per liter of water in the cells or serum.

2 The direct determination of the Donnan ratio is accomplished in the following steps

a Separate cells and plasma by centrifuging

b Determine the concentration of the ion in the cells and plasma

c Determine the water concentration in the cells and plasma by drying weighed volumes at 110 C (see estimations of water and solids)

d. Calculate the Donnan ratio according to the equation

$$r \text{ the Donnan ratio} = \frac{\text{mEq/l cells}}{\text{mEq/l plasma}} \times \frac{\text{ml water/1000 ml plasma}}{\text{ml water/1000 ml cells}}$$

3 The indirect determination of the Donnan ratio is accomplished with measurements on whole blood and plasma

a. Determine the hematocrit i e ml packed red cells/100 ml blood.

b Determine the concentration of the ion in whole blood and in plasma

c Determine the water content of whole blood and plasma by drying weighed known volumes at 110 C (see estimation of water and solids)

d. Estimate the concentration of ion in plasma mEq/l

e Estimate the concentration in whole blood mEq/L

f Compute the concentration in cells according to the equation

$$\text{mEq/l cells} = \frac{\text{mEq/l whole blood}}{\text{Hematocrit/100}} - \frac{\text{mEq/l plasma} \times (100 - \text{Hematocrit})}{\text{Hematocrit}}$$

g Compute the Donnan ratio

$$r \text{ the Donnan ratio} = \frac{\text{mEq/l cells}}{\text{mEq/l plasma}} \times \frac{\text{ml water/1000 ml plasma}}{\text{ml water/1000 ml cells}}$$

Composition of Normal Arterial Human Blood

For some purposes a table of some of the properties of normal human arterial blood is useful. Table 19 has been compiled from data accumulated at the Harvard Fatigue Laboratory

Temperature Effects on Base Bound by Plasma Protein

Reference

1 Stad e W C Austin J H and Robinson H W The Effect of Temperature on the Acid Base Protein Equilibrium and Its Influence on the CO Absorption Curve of *Whole Blood* True and Separated *Serum* *J Biol Chem* *66* *901 9 0* (Dec) 19 5

2 Van Slyke D D Hastings A B Hiller A and Sendroy J Jr Studies of Gas and Electrolyte Equilibria in Blood XIV The Amounts of Alkali Bound by Serum Albumin and Globulin J Biol. Chem 79 ,69 ,80 (Oct) 1928

Calculation

The base bound by plasma protein, expressed as BP in mEq/l is a function of

a Concentration of plasma protein P expressed in gm/l

b pH

c pI the pH of minimal base binding

d. Buffer value expressed by a constant 0 104 in the case of normal man

The e juation at 38 C according to Van Slyke Hastings Hiller and Sendroy is

BP = (0 104) × (P) × (pH − 5 08)

At this temperature pI has the value of ⌄08

The effect of temperature on pI according to Stadie Austin and Robinson is about −0 0 per 1 C that is

Δ pI = −0 0‿ (Observed T − 38 C)

Hence the general equation for evaluating BP tak ng temperature into account is

BP = (0 104) × (P) × pH − (5 08 − Δ pI)

By substitution this becomes

BP = [(0 104) × (P)] − [08 − 0 0? (t − 38 C)]

where t = observed temperature

TABLE 19

COMPOSITION OF NORMAL ARTERIAL HUMAN BLOOD

Averages based on over 100 analyses at the Harvard Fatigue Laboratory with healthy young men as subjects)

MEASUREMENT	CELLS	PLASMA	WHOLE BLOOD
pH	19	7 40	
Volume ml/l	448	55	1000
Sp Gravity	1 100	10 7	1 060
Water ml/l	720	938	840
Total Solids gm/l	380	89	2 0
Protein gm/l	356	70	100
Glucose gm/l	0 9	1 1	1 0
Sod um mEq/l	14 9	140	83 7
Potass um mEq/l	93 7	5 3	44 9
Calcium mEq/l	0 0	5 0	2 6
Magnes um mEq/l	0	? 0	° 0
Total cat ons mEq/l	110 6	15 3	133 2
Chlo de mEq/l	54	104 6	82 0
B carbonate mEq/l	15 3	5 1	0 7
Lactate mEq/l	0 7	1 4	1 1
Inorganic Phosphorus mEq/l	1 1	°	1 7
Inorganic Sulfate mEq/l	0 6	0 6	0 6
P oteinate mEq/l	41 7	17 4	°8 2
Total an ons mEq/l	113 3	151 3	133 9

SECTION VI

PHYSIOLOGICAL MEASUREMENTS (Continued)

C ESTIMATION OF RESPIRATORY GASES

1 The Haldane Apparatus for Measuring Carbon Dioxide and Oxygen

Reference

Haldane, J S and Priestley, J G *Respiration*, Oxford 1935, Clarendon Press

Principle

The CO_2 in a known volume of air is absorbed with KOH, and the O with a strong reducing solution The remainder of gas is considered to be all N

Apparatus

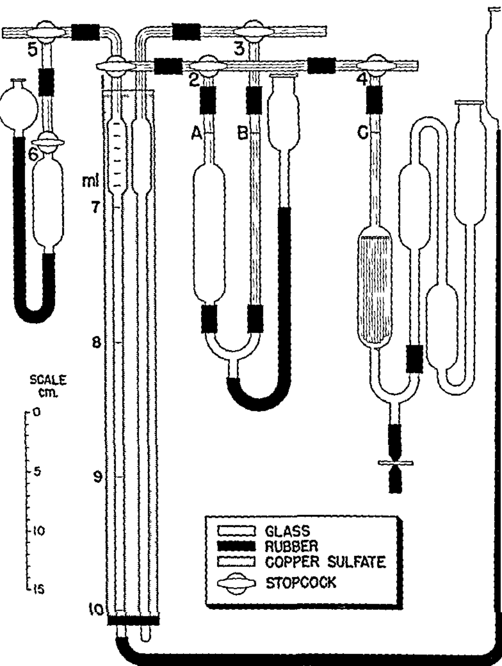
1 Haldane gas analyzer (See Fig 41)
2 Gas sampling tubes Oiled syringes may be used if storage time is only a few hours
3 Pipe cleaners for cleaning apparatus
4 50 ml syringes for handling reagents and cleaning apparatus
5 Dropping bottle for dilute H_2SO
6 Small lengths of pressure tubing for cleaning apparatus

Reagents

1 Dilute H SO approximately 1 ml conc H_2SO_4 diluted to 100 ml with water
2 KOH approximately 14 gm dissolved in 100 ml water
3 Litmus paper for detecting acid or alkali in the wrong places
4 Oxygen absorbent In a screw topped bottle place about 8 gm of sodium hydrosulfite (Lycopon Na_2SO) 1 5 gm sodium anthraquinone beta sulfonate and a small crystal of ferric chloride Just before the apparatus is to be made ready for use add approximately 50 ml of 14% KOH which has been warmed to about 50 C Solution is complete in a few minutes Throughout all manipulation of the absorbent unnecessary exposure to air must be avoided It is wise to weigh out about 6 bottles of dry reagents at a time since 50 ml of absorbent will be enough for about 50 analyses
5 Stopcock grease mercury and copper sulfate crystals

Procedure—Calibrating Burette

1 This is necessary before a new burette is used since the bore is not necessarily uniform It need not be repeated unless structural alterations are made in the burette
2 Fuse a one way stopcock with a fine tip on the lower end of the burette (Most companies ship burettes with stopcock attached.)
3 Clean the burette with sulfuric acid dichromate mixture



HALDANE APPARATUS

C E GORDON

Fig 41.—Haldane's apparatus for measuring oxygen, carbon dioxid and nitrogen in samples of air

4 Rinse thoroughly with water and finally with dilute sulfuric acid and allow to drain for 10 minutes
5 With the short limb of Stopcock 1 to the left and the long limb open to air fill the burette with mercury exactly to the top of the stopcock bore Hang a thermometer right next to the burette
6 Into a weighed 25 ml Erlenmeyer flask deliver mercury through the fused bottom stopcock until the mark 7 00 is exactly reached by the upper part of the mercury meniscus Measure the temperature
7 Reweigh the flask
8 Now deliver mercury from the bottom stopcock into the flask until exactly 7 100 is reached measure the temperature and weigh again
9 Repeat this for 7 200 7 300 7 400 and so on to 10 000
10 The calibration is now calculated by the following steps
 a. List the burette readings (Column 1)
 b List the corresponding temperatures and weights of Hg (Columns 2 and 3)
 c From 7 0 on calculate the increment in weight for each 0 1 ml division on the burette (Column 4)
 d List the density of mercury i e, gm of Hg per ml at given temperatures (Column 5) from table 20
 e Calculate the increment in volume for every 0 1 increase in burette reading by dividing every number in Column 4 by the corresponding number in Column 5
 f Calculate the actual volume of the burette at every 0 1 division by determining the actual volume at 7 0 (i e weight of Hg at 7 0 divided by density of Hg at the temperature of the burette when 7 0 was weighed) and adding to it successively each number in Column 6
 g The final answer in a Haldane analysis is expressed as ml of gas per 100 ml of initial sample Hence it is now necessary to calculate all burette readings on the basis that burette reading 10 0 represents exactly 10 00 ml. This is done by multiplying all readings in Column 7 by the factor

$$\frac{10\ 000}{\text{Column 7 reading for 10 000}}$$

 h. The correction factor for each burette reading is now calculated (Column 9) This correction represents the number which has to be added to any given burette reading in order to give its value correctly on the basis of 10 000 = exactly 10 ml
 i The table of correction factors is placed on a card which is mounted for use on the Haldane apparatus which has been calibrated
 j Steps 10a through 10h are illustrated in Table 21 which presents the actual calibration of a new burette

TABLE 20

DENSITY OF MERCURY AT VARIOUS TEMPERATURES

C	0 0	0 2	0 4	0 6	0 8
	gm Hg PER ml AT DIFFERENT TEMPERATURES				
20	13 5462	13 5457	13 5452	13 5448	13 5443
21	5438	5433	5428	5423	.5418
22	5413	5408	5403	5399	5394
23	5389	5384	5379	5374	.5369
24	5364	5359	5354	5350	5345
25	13 5340	13 5335	13 5330	13 53 5	13.53 0
26	5315	5310	5305	5301	5296
27	5 91	5286	5281	5278	.5 47
28	5 66	5261	.5256	.5252	5 [illegible]
29	5242	.5237	5232	5227	

TABLE 21

SAMPLE CALIBRATION OF A HALDANE BURETTE

1 BURETTE READING UNITS	2 TEMP OF BURETTE C	3 WEIGHT OF Hg gm	4 INCREMENT IN WEIGHT gm	5 DENSITY OF HG gm/ml	6 INCREMENT IN VOLUME ml	7 ACTUAL VOLUME ml	8 VOLUME ON BASIS 10 0 = 10 ml	9 CORRECTION FACTOR UNITS
7 0	4 5	98 0339		13 535		7 43	7 004	+4
7 1	3.5	99 4474	1 4135	13 5376	0 1044	7 347	7 105	+5
8 0	4 6	111 9589	1 3815	13 5350	0 10 0	8 7	8 000	0
8 1	24 6	113 36 4	1 4035	13 5350	0 1037	8 375	8 099	-1
8 9	4 7	124 5315	1 3935	13 5347	0 10 9	9 00	8 897	-3
9 0	24 8	1 5 9389	1 4074	13 5345	0 1040	9 304	8 998	-2
9 9	25 1	138 5453	1 3808	13 5337	0 10 0	10 236	9 900	0
10 0	5 1	139 9565	1 4112	13 5337	0 1043	10 340	10 000	0

Th cor ection factor i the numbe to be added in the third decimal place to the actual bu ett ading in order t make it ep esent the actual volum on the basi that bu ette reading 10 0 i exactly 10 ml Exampl Burette ading 8 957 Corre tion Facto from Column 9 2 Corrected reading 8 955

Procedure—Setting Up and Preparing Apparatus for Use

1 Install the burette and dummy burette in the water jacket put a few crystals of CuSO in the bottom and fill with water to ½ inch of the top The tube of an air bubble either hand dr ven or from a pressure pump should be installed at the bottom of the jacket The dummy burette should contain 1% H_2SO up to about 8 ml.

Remove all stopcocks and clean out their tubes and cores

3 With stopcocks still out fill the me cury reservoir with mercury so that the band bulb is about 1/3 full when the levels of mercury in the bulb and burette are the same and the mercury in the burette is almost up to the Stopcock 1 Suck 3 drops of 1% H SO in on top of the mercury column Now hang the hand bulb on the right hand rack and pinion low down so that the mercury is at about 10 ml in the burette

4 Grease and insert Stopcock 1 leaving it in the full off position (not connected with any part of the machine or outside a r)

5 With Stopcocks 2 3 and 4 taken out and the KOH bulb in the position shown add 14% KOH to the bulb until the KOH level is at A and B and the bulb is about 1/4 full.

6 Grease and insert Stopcocks 2 and 3 leaving them in the full off pos t on

7 With Stopcock 4 taken out add 14% KOH to the far right hand glass tube until its left hand bulb is full and the KOH is almost to the bottom of its r ght hand bulb Th s is necessary to protect oxygen absorbent from atmospheric oxygen

8 Fill a 50 ml syringe with oxygen absorbent up to about the 40 ml mark Unscrew the p nch clamp on the rubber nipple at the bottom of the middle U tube and nsert the nozzle of the syringe Force oxygen absorbent slowly up into the U tube until it is about 2 cm above C Air will now be trapped in the right hand bulb of this U tube and oxygen w ll be absorbed. During this period lasting about 1 minnutes keep the level of absorbent at about 2 cm above C by slowly pushing it in as necessary from the syringe When the rapid absorption of oxygen has ceased clamp off the rubber n pple at the bottom and remove the syr nge The abso bent should now be approximately 2 cm above level C Oxygen absorpt o is not complete for ½ hour

9 Grease and insert Stopcock 4 leaving it n the full off pos tion

10 Before an analys s can be performed the whole apparatus from Stopcock 1 to levels A and C must be full of nit ogen This is accompl shed by ca rying out one analys s as described below but without any measurements

Procedure—Taking a Sample of Gas

1 The gas sampling tube is fixed near the upper left hand corner of the machine and is connected as shown to the burette by means of rubber tubing through a 3 way stopcock of small bore For field use, syringes are best and the following description applies only to them.

2 *Stopcocks 1 and 5 are opened so as to connect the burette with outside air but not with the rest of the apparatus or the gas sampling syringe*

3 With the right hand *mercury leveling bulb is detached from the rack* and is carefully raised until the mercury in the burette almost reaches Stopcock 1 It must never enter Stopcock 1

4. Stopcock 5 is turned with the left hand so as to connect the sampling tube with the burette but not outside air and the mercury bulb is lowered 5 or 10 cm so as to exert a negative pressure on the sampling tube and prevent contamination of the sample in Step 5

5 The pinch clamp on the nozzle of the syringe is loosened with the left hand and with the right the mercury bulb is lowered until the burette reads about 10 Stopcock 5 is turned so as to connect the burette with outside air but not the sampling tube (Keep the left hand on Stopcock 5 for smooth and accurate sampling *No pressure is required on the barrel of the syringe unless the* sampling tube has been poorly oiled)

6 Smoothly raise the *mercury bulb When the column is approaching Stopcock 1* turn Stopcock 5 to connect the burette with the sampling tube but not with outside air and simultaneously lower the mercury bulb until the column stands at about 10

Repeat Step 6 once more and hang the mercury bulb on the rack and pinion so that the mercury level is 10 0 or slightly less.

8 Turn Stopcock 1 to the full off position and close the pinch clamp on the sampling tube

Procedure—Analysis of Sample

1 For analysis the machine must have nitrogen in it from Stopcock 1 to A and C and the fluid levels should be at A B and C Both of these requirements will have been fulfilled by a previous analysis

2 Turn Stopcock 3 to be open to dummy burette KOH and outside air

3 *Turn Stopcock 2 to be open to burette and KOH but not outside air*

4. Turn Stopcock 1 to be open to burette and KOH but not outside air

5 Rapidly adjust the KOH bulb up and down until the fluid level is at B and turn Stopcock 3 to connect with dummy burette and KOH but not outside air

6 Adjust fluid level to be exactly at A by moving the rack and pinion. Bubble air through the water jacket (The purpose of the dummy burette is to compensate for possible changes in temperature or barometric pressure during the course of an analysis. This can be seen from Fig 41 If the fluid levels are at *A* and *B* and the samples of gas in the burette and the dummy burette are at external barometric pressure *as they will be if all the steps above are followed carefully* any change of temperature in the apparatus or any change of pressure will affect burette and dummy burette equally The gas in the burette is thus put in a condition which makes any change in its volume due *solely* to the removal of CO_2 or O_2)

7 Read the total volume, V at the top of the mercury meniscus Check *reading after* readjusting levels A and B Correct V *from the correction chart*

8 *In order to absorb CO₂ raise the mercury bulb until the mercury is in the enlarged portions of the burette* Raise and lower the bulb 6 8 times *keeping the mercury oscillating between about 2 and 6 ml*

9 Carefully hold the mercury bulb next to the rack and pinion with the level at about 6 ml and slowly lower it until the KOH is about 1 cm below level A Hang the bulb on the rack and pin on

10 Adjust levels A and B bubble air through the water jacket and read the burette V at the top of the mercury meniscus

11 Repeat Steps 8 and 9 with 4 flushes into KOH

12 Repeat Step 10

13 If the second reading of V is not with n 0 001 of the first repeat Steps 8 9 and 10 until checks are obtained (If V becomes progressively smaller there is probably a leak in Stopcock 1 or) Correct V from the correction chart

14 To absorb O turn Stopcock 2 to connect the burette with abso ber but not with KOH

15 Using the same technique as in Step 8 rinse the gas into the absorbent about 8 times

16 With the r ght hand holding the mercury bulb bring the absorber carefully up to approximately C and hold it there while with the left hand Stopcock 2 is turned to onnect the burette w th KOH but not absorbent

17 In order to remove O from the gas between Stopcock 2 and A r nse gas into KOH ° 3 times

18 Bring fluid level to approximately A and turn Stopcock to connect burette with absorbent but not KOH

19 Repeat Steps 15 16 and 17 once

20 Rinse into absorbent finally about 3 t mes ha g the bulb on the rack and pin on and bring the level exactly to C

1 Turn Stopcock 2 to connect burette with KOH but not absorbent and adjust KOH levels exactly to A and B and bubble a r through the water jacket Read the burette V at the top of the mercury men scus

2 Repeat Steps 14 15 16 17 0 and 21 V must check to at least 0 001 ml (If it drops progressively there are probably leaks in the stopcocks or else the absorbent is inefficient) Correct V from the correction cha t

23 Prepare for the next analys s by opening Stopcock 3 to dummy burette and KOH but not outside air readjusting KOH levels to exactly A and B closing Stopcock 3 to connect with dummy burette and KOH but not outside air readjusting levels A and B and opening Stopcock 1 to connect with outside air but not to the machine

4 When leaving the apparatus after a series of analyses turn Stopcocks 1 2 and 4 to full off pos t on and turn Stopcock 3 so that it connects dummy burette outside air and KOH The above precautions are necessary to prev nt changes in temperature or barometric pressure sucking over the solut ons into the wrong parts of the apparatus

Procedure—Cleaning the Apparatus

1 When KOH or oxygen absorbent has been sucked into Stopcocks 4 . 1 or sometimes even further cleaning with water and finally dilute sulfuric acid is necessary If there is a susp cion of such a misfortune remove the stopcock and nsert a piece of red l tmus paper If it turns bl e cleaning is necessary because alkali is present

Open taps 1 and 4 to outs de a r but not to burette KOH or absorb nt and disconnect Stopcock 5

3 Attach a piece of rubbe tubing to the glass tubing to the right of Stopcock 4

4 Using a large syringe force 100 ml of water through the whole line of glass tubing nto a beaker held under the outs de air connect on of Stopcock 1

5 Follow this witl 50 ml of dilute sulfur c ac d and r move excess fluid by pumping a r through w th the empty syringe

6 If it is suspected that alkali has reached the burette remove Stopcock 1, run the mercury right up to the stopcock seat and test with litmus paper If there is alkali, replace Stopcock 1 turn it to connect burette with outside air but not machine and rinse twice by sucking dilute sulfuric acid all the way beyond 10 and discarding it through the stopcock Leave 2 3 drops of dilute sulfuric acid in the burette
7 Remove all stopcocks wash them in hot water clean them with a pipe cleaner dry them, *grease them and put them back in the apparatus*
8 Run an analysis without measurements in order to fill the apparatus with nitrogen.

Calculations

$$\text{ml CO per 100 ml sample} = \frac{V \text{ corrected} - V_1 \text{ corrected}}{V \text{ corrected}} \times 100$$

$$\text{ml O per 100 ml sample} = \frac{V_1 \text{ corrected} - V \text{ corrected}}{V \text{ corrected}} \times 100$$

$$\text{ml } N_2 \text{ per 100 ml sample} = \frac{V \text{ corrected}}{V \text{ corrected}} \times 100$$

Example

	READING	BURETTE CORRECTION	CORRECTED READING
V	9 897 9 897	0	9 897
V	9 643 9 64°	+2	9 644
V	7 901 7 900	+5	7 905

$$\text{ml } CO_2 \text{ per 100 ml sample} = \frac{9\,897 - 9\,644}{9\,897} \times 100 = \frac{25\,3}{9\,897} = 2\,56$$

$$\text{ml O per 100 ml sample} = \frac{9\,644 - 7\,905}{9\,897} \times 100 = \frac{173\,9}{9\,897} = \text{[illegible]}$$

$$\text{ml N per 100 ml sample} = \frac{7\,905}{9\,897} \times 100 = 79\,87$$

$$CO_2 + O_2 + N_2 \text{ should} = 100$$

Precautions—Reagents

1. One filling of oxygen absorbent suffices for about 50 estimations When it is be coming weak scum forms at level C and V checks are hard to obtain It is then *time to change the reagent*
2 One filling of KOH will last for about 1000 estimations

***Precautions*—Calibrating Burette**

1 There are no short cuts in accurate calibration Each burette should be cal ibrated twice or until good agreement is obtained

Precautions—Setting up Apparatus

1 A chief source of systematic errors is improper lubrication of stopcocks When properly seated they show no *air bubbles in the grease layer between the core* and the shell of the stopcock and the capillaries are free of grease

Precautions—Taking *Gas* Samples

1 It is essential to have the gas in the burette at external barometric pressure be fore connecting burette with KOH through Stopcocks 1 and 2 Otherwise KOH will inevitably be sucked over *or else will be pushed far enough down to absorb*

CO before V can be read A properly oiled syringe will avoid this but if there is any b nding during the taking of samples set the mercury at about 10 for the final step leave Stopcock 1 as it is and open Stopcock 5 to burette and outs de air before closing Stopcock 1

Precautions—Analysis of Sample

1 The commonest error is suck ng KOH oxygen absorbent or both through Stop cocks 2 and 4 This can be avoided by

a Avoiding jerky abrupt movements

b Slowing down whenever mercury KOH or absorbent approach or leave con strictions in the apparatus

c Never moving the mercury bulb unless one is sure that the stopcocks are turned in the correct manner

d During absorption of CO or O making sure that the mercury in the burette does not fall below 7 0

All read ngs must ch ck to within 0 001 ml When successive readings fall pro gressively

a In the case of V_0, there is either a leak in one or more stopcocks or else CO is being absorbed from the sample by KOH somewhere in the system

b In the case of V there is either a leak in one or more stopcocks or else oxygen absorbent has reached to or beyond Stopcock 2

c In the case of V there is either a leak in one or more stopcocks or else the oxygen absorbent has become inefficient

3 Erratic read ngs usually come (a) from errors in technique (b) if the burette does not contain acidulated water (c) if grease or mercury act as a ball valve somewhere in the apparatus

4. Be sure to follow Steps 23 and 24 carefully in order to avoid trouble in subse quent analyses

Precautions—Cleaning the Apparatus

1 Avoid using alcohol ether or other organic solvents They are hard to remove completely from the system are in part d ssolved in the reagents have high vapor pressure and give very errat c results

Precautions—Calculations

1 If the volume of dilute sulfuric acid in the burette amounts to more than 0 005 ml it must be taken into account in the calculations s nce it takes up volume that would otherwise be occup ed by gas It is wise to adopt the follow ng routine

a After the apparatus has stood undisturbed for at least an hour read the top of the mercury men cus and the bottom of the dilute sulfuric ac d meniscus

b V V and V are read as usual

c In all calculations the d fferences between V_0, V and V are calculated as usual However instead of dividing by V one divides by (V − amount of dilute sulfur c acid) If d lute sulfuric acid is less than 0 005 it is neglected

2 Before p oceeding to a series of analyses it is wise to analyze a sample of pure outdoor air The percentage of CO should be 0 03 ± 0 01 and the percentage of O should be 20 93 ± 0 0 If one cannot achieve th s accuracy there is no use pro ceeding to analysis of unknown samples More practice or else cleaning of the ap paratus w ll be necessary

3 In satisfactory duplicate samples of exp red air analysis for CO must agree to within 0 03 percent and analyses for O must agree to within 0 04 percent Unt l the operator can achieve these limits he is unreliable

2 The Scholander Micrometer Gas Analyzer

Reference

Scholander, P F Analyzer for Accurate Estimation of Respiratory Gases in One Half Cubic Centimeter Samples *J Biol Chem.* *167* 235 250 (Jan) 1947

Principle

The *present analyzer permits the* determination of carbon dioxide oxygen and nitrogen in 0 5 ml amples with an accuracy of ±0 015 volume percent. It will handle directly samples containing from zero to over 99 percent absorbable gases. The analys s requires 6 to 8 minutes A gas sample is introduced into a reaction chamber, connected to a micrometer burette and is balanced by means of an *indicator drop in a capillary* against a compensating chamber Absorbing fluids for carbon dioxide and oxygen can be tilted into the reaction chamber without causing any change in the total liquid content of the system During absorption of gas mercury is delivered into the reaction chamber from the micrometer burette so as to maintain the balance of the gas against the compensating chamber Volumes are read *in terms of micrometer divisions* The rinsing fluid and absorbents are accurately adjusted to have the same vapor tension

Apparatus (See Fig 42)

1 Reaction Chamber Unit This part of the apparatus consists of a compensating chamber (A) a reaction chamber (B) and two side chamb rs (C and D) for storing absorbents for carbon dioxide and oxygen respectively

The compensating chamber or thermobarometer (A) can be closed with a stop cock (S 1), the lower end of which is ground to fit into the neck of A Between analyses S 1 is kept in a ground glass receptable (G) A 1 mm bore capillary, with the upper end ground to a wide angled funnel, intrudes into the bottom of the compensating chamber The capillary is ring marked in the middle The lower end of the capillary bore is coarsely surface ground for 2 mm and is covered with a Clarite coating The upper part of the compensating chamber is also made hydrophobic with a Clarite coating The somewhat unconventional scheme of running the indicator drop vertically is correct as long as the weight of the drop remains constant during the analysis The intruded capillary insures that no acid can trickle down and add to the weight of the drop The lower end of the reaction chamber unit is ground to fit the micrometer burette unit The side arms C and D of the reaction chamber are furnished with capillaries for filling The ends are stoppered with tight fitting solid vaccine bottle rubber stoppers (E and F)

2 Micrometer Burette Unit A micrometer burette H is attached to a 3 way stop cock (S 2) the upper limb of which is ground to fit the lower end of the rea n chamber unit The side limb is connected with a mercury leveling bulb (I) through rubber or plastic tubing A 5 mm thick round wooden dowel 10 cm long is fitted to the knurled ratchet on the handle of the micrometer by means of a 5 cm length of rubber tubing

3 Water Bath The reaction chamber unit is immersed in a water bath which can be made of a gla s bottle with the bottom cut off A one hole rubber s opper is inserted in the neck of the bottle and the upper limb of the micrometer burette is inserted through the hole The rubber stopper should be short enough to permit rocking the apparatus by tapping the micrometer burette unit The water bath rests on a ring which can be tilted on a friction bushing The position of the ring is controlled by means of an attached handle (J) The water level reaches to th neck of (A) so that the gas in the thermobarometer is totally immersed. The water bath is continuously stirred by bubbling air from the bottom A 50 ml centrifuge tube (K) is suspended from the edge into the water bath

SCHOLANDER GAS ANALYZER

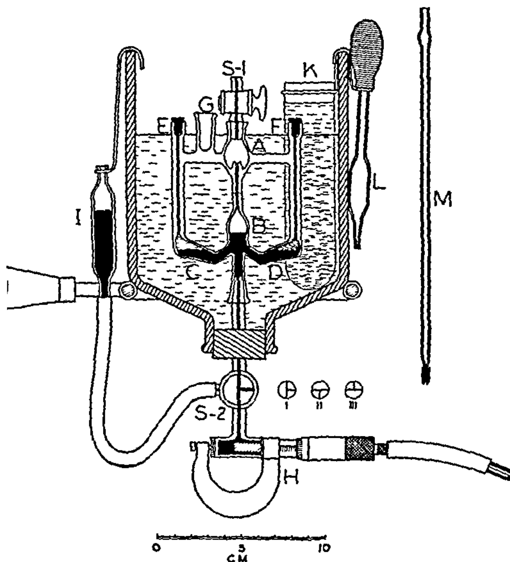


Fig 4 —Analyze f r accurate stimation f espiratory g ses i 0 5 ml ample *A* C m pe sating chambe (th rmo-ba om t r) *B* reaction chamber *C* side a m for ca bon dioxide ab rber *D* side a m for oxygen abso ber *E* and *F*, solid vaccin bottle stoppers *G* re ceptacle fo top-cock *S 1* *H* microm t r bu ette *I* leveling bulb *J* handle f r tilting of pparatus *K* tub fo sto ing a id insing solution *L* pipette for rinsing acid *M* transfe pipett The heavily d awn lowe end of the capi lary at *B* indicates where th capillary i surf ce gr und and oated with Clarite.

and holds the acid rinsing solution described below A 5 ml pipette (L) furnished with a rubber bulb is suspended on the outside of the water bath. A suction line with a 15 cm long fine tipped capillary glass tube is used for sucking off waste fluids It is desirable to furnish the apparatus with a device to shake or vibrate it during the absorption of oxygen A small motor with a crankshaft imparts motion to the micrometer burette through a rubber band attached by means of a metal ring to the crank shaft The rate should be about 10 r p s and the amplitude about 1 cm at the crank shaft

Reagents

1 Acid Rinsing Solution In 400 gm distilled water dissolve 7° gm anhydrous sodium sulfate (Na SO) 1 ml conc H SO (delivered from a calibrated tuberculin syringe) and 21 ml glycerin (delivered from a calibrated syringe) *Before use dissolve 50 mg potassium dichromate (K Cr O) in 100 ml of the mixture and* store in water bath of apparatus

2 Carbon Dioxide Absorbent In 100 gm of water dissolve 11 gm potassium hydroxide (KOH) and 40 mg potassium dichromate (K_2Cr O) Store in a syringe fitted with a No 24 needle

3 Oxygen Absorbent Prepare (a) a solution of 100 gm water and 6 gm potassium hydroxide and (b) a powder mixture of 10 gm sodium hydrosulfite (Na_2S_2O Lycopon) and 0 1 gm sodium anthraquinone beta sulfonate For use place 0 6 gm of powder (b) in an 8 ml vial add 5 ml of solution (a) close with finger dissolve anaerobically and transfer immediately to a 5 ml syringe fitted with a No 24 needle

4 Clarite Coating Solutions *Clarite X is a thermoplastic resin commonly used for mounting histologic sections* Two strengths of solution are required (a) dissolve 3 gm Clarite Y in 2 ml of toluol (b) dissolve 1 gm Clarite X in 4 ml of toluol

5 Petrolatum White commercial petrolatum is used

Procedure—*Assembling Apparatus*

1 First insert the rubber stoppers and indicator band (1 1 5 *mm* binding tape) on the analysis capillary

2 Then with the lower joint dried and the female part very lightly greased with petrolatum fix the upper part of apparatus back in place and jam it on

3 Put water (room temperature) *into the jar up to the neck of the compensating* vessel Connect to the leveling bulb and let the mercury up into the analysis capillary

4 Leaving the apparatus connected with the leveling bulb insert an *empty* CO_2 absorbing syringe via the needle through the middle of the rubber cork and remove the air Hg will follow up into the syringe

5 Remove the needle from the stopper at the same time pressing down the whole apparatus so that it does not disconnect at the ground point

6 Repeat on the other side

Then charge the respective sides with the corresponding absorbents (all bubbles must be excluded)

8 If the lower joint traps any *air bubble remove the same by working the rinsing* acid through it

Procedure—Disassembling and Coating of Capillary

1 Suck out all the water *from* the water bath and remove any rinsing acid from the apparatus Lower the mercury below the ground point by lowering the reservoir Lift off the upper part *of the apparatus from the ground point*

- Holding it over a beaker remove the plugs so that the Hg and absorbent empty into a beaker Wash thoroughly under a water tap and remove most of the water by suction
3 Pour toluol into the compensating chamber holding the finger in the lower end rinse through the middle with toluol Repeat wash thoroughly with acetone and dry by suction Coating solution is 10 to 0% Clarite in toluol
4 Draw out a glass tube into a fine capillary which will reach the ground ends ins de of the capillary of the analys s apparatus both from above and below Attach a rubber tube to the glass tube and fold the rubber tube then squeeze it together
5 Place the end of the glass capillary in Clarite and relax the squeezing so that the Clar te fills the capillary Then let go the fold of the rubber tube to prevent air from being sucked in when capillary is taken out of Clarite
6 Wipe outs de of the capillary Fold rubber tube over and insert glass capillary into upper ground end of analysis capillary Squeeze out drop (about ° mm) of Clarite and withdraw capillary
7 Move the drop of Clarite down the analysis capillary until it reaches the end of the ground part (e about 1 cm) by placing finger on the opening of the com pensating chamber and pressing Similarly move the drop back and withdraw it by suction through its own capillary pipette rinsed with toluol to remove Clarite still there D y with vacuum.
8 The other end of the capillary is coated in the same way from the other end The middle third of the capillary must be untouched by the coating solution
9 A sl ght suct on s applied to the apparatus overnight
10 The upper half of the compensating chamber is also coated with Clar te so as to protect the joint above from being wetted by water Clarite must be removed from this joint by toluol swabbing if it acc dentally gets on it The Clarite is swabbed on to the upper half by a plug of cotton on the end of a match

Procedure—Washing Out Apparatus

1 F ll the thermobarometer up to the neck w th ac d rins ng solution
2 With Stopcock in position III draw the rinsing solution up and down past the side arms by moving the leveling bulb (not the micrometer)
3 Suck out the rins ng solut on and epeat the i se unt l the solution remains yellow This takes two to four times
4 Connect the micrometer with B (pos tion II) and adjust mercu y to the top of the capillary
5 Leave the mometer half full of acid rinsing solution

Procedure—Analysis of Samples

1 Gas is contained in the usual sampl g tube (Ba c oft type) w th tap and Hg reservo r From a syringe squirt a small drop of Hg nto the bottom of the tube just above the tap Hold a d y transfer p pette (M) vertically and press the rubber t p firmly against the top of the tube Open the tap caut ously so that the Hg d op is gently blown up into the pipette bulb and allow the gas to bubble past it for a few seconds W thdraw the pipette The Hg d op starts to fall slowly down the tube Before it has had time to get far fit the rubber tip on to the top of the cap llary in the compensat ng vessel (A) there being a layer of rins ng solut on a few mm thick to protect it from the outs de air

Screw the micrometer (H) out until the gas men scus reaches the lower mark turn the stopcock (pos tion II) to connect the micrometer and bulb and adjust the mic ometer to zero Th s s e o reading M

3 Turn the stopcock to connect the *micrometer with the analysis chamber (position* I) and screw the micrometer out to about 15 to 20 on the scale Screw micrometer back a few mm so that the Hg drop moves up thereby indicating slight positive pressure in chamber In this way no acid will leak in when the transfer pipette *is removed.*

4 Screw in about 2 mm of the rinsing fluid into the capillary and suck away all the rest of the rinsing fluid from the compensating chamber (A) Screw the acid drop down until the lower meniscus of the drop coincides with the lower *mark on the capillary*

5 Insert the tap of the compensating chamber and close the tap (S 1)

6 Readjust the drop meniscus accurately and repeat until the micrometer reading is constant (M)

7 Tilt the apparatus *downward to the carbon dioxide absorbent side (C) admitting* a small amount of the latter into the analysis chamber at the same time screwing in the micrometer to compensate for the downward movement of the acid drop in the capillary occasioned by the carbon dioxide absorption (tap with finger to absorb *carbon dioxide) When the acid drop is stationary readjust* to mark and read micrometer again (M)

8 Again tilt downwards on the oxygen absorbent side (D) drawing in similarly rather more of the latter again screwing in the micrometer to take care of the oxygen absorption.

9 Then switch on the motor shaker until the absorption is finished the acid drop being kept in a safe part of the capillary by the continuous screwing in of the *micrometer*

10 Allow about 2 minutes more for the temperature rise (due to the oxygen + Na_2S_2O reaction) to disappear

11 Then readjust the drop to the mark and read micrometer (M)

12 Open the compensating chamber to the air by turning the key of the tap (S 1) and after doing that transfer the tap to its sockets (G)

13 Screw micrometer in until the top of the absorbing solution in the chamber coincides *with the bottom mark in the capillary Read micrometer* again (M) It should be zero

14. Turn the 3 way tap to connect the leveling bulb with the absorption chamber (position *III with S 2) and eject the absorbing solution into a vacuum junction* tip not allowing the absorbing solution to spill into the compensating chamber

15 Rinse twice with the acid solution as described at the beginning of the procedure

16 To recharge the side arms (E and F) with respective absorbents puncture *the rubber stoppers through their central hole* taking great care not to introduce any gas bubbles The side arms should not be filled more than ½ full with the absorbents

Calculation

1 Carbon dioxide ml per 100 ml original sample dry =

$$\frac{M - M}{M} \times 100$$

2 Oxygen ml per 100 ml original sample dry =

$$\frac{M - M}{M_2} \times 100$$

3 Nitrogen ml per 100 ml original sample dry =

$$\frac{M_3}{M} \times 100$$

Example

M was 9 888 M was 9 5 5 M was 8 000
M_4 and M_4 were 0 00 which showed a satisfactory analysis
Carbon diox de ml/100 ml dry =

$$\frac{9\,888 - 9\,5^{\circ}5}{9\,888} \times 100 = 3\,671$$

Oxygen ml/100 ml dry =

$$\frac{9\,5\;5 - 8\,000}{9\,888} \times 100 = 15\,423$$

Nitrogen ml/100 ml dry =

$$\frac{8\,000}{9\,888} \times 100 = 80\,906$$

Precautions

1 *Apparatus* The micrometer must be all steel the plunger uniform from one end to the other as measured by another standard micrometer the screw without any play and the lap fit at the plunger bearing perfect as determined by visual in spection with the plunger screwed level The glass portion of the glass metal junction in the mi rometer burette unit has to be ground perfectly flat in order to make a mercury tight junction

2 *Transfer Pipette* The tip of this is critical and must conform to the directions given. *If the tip becomes plugged clear it with a fine wire or hold the rubber* tip of the pipette against the nozzle of a 5 ml syringe and press air through the tip Between samples the mercury drop and the drop of the ac d rinsing solution are sucked out of the pipette by appl cation of the suction tip to the rubber tip on the pipette

3 *Analysis* The most critical part of the analysis is correct delivery of the gas sample avoiding suction on the micrometer burette A proper line up between the bore of the transfer pipette and the bore of the reaction chamber capillary is eas ly accomplished by means of the funnel ground capillary and conical rubber tip to match a most valuable improvement of the original model contributed by Mr Warner Love of the Department of Zoology Swarthmore The transfer pipette must be kept clean The easiest way to avoid clogging is to make use of a long and relatively wide bore for the air resistance in the tip instead of a short and very fine one

a The reason for not setting the micrometer at zero exactly at the beginning of the analysis is as follows The rubber stoppers E and F are elastic and a trace of elasticity is l kewise derived from unavoidably trapped minute air bubbles Hence gross changes in mercury levels between the reaction chamber and the side arms will sl ghtly affect the total volume of the apparatus When the mercury level is lowered from the mark on the capillary to half way down the reaction chamber during introduction of the gas sample there is consequently a minute decrease in the total liqu d capacity of the apparatus The mag nitude of th s shrinkage is easily determined by reading the gas meniscus at the mark with the mercury almost to the mark then letting in all absorbing fluids from both side arms drawing down the mercury from the capillary and readjust ng the gas meniscus to the mark The reading will then be close to 0 5 division higher than before Setting the micrometer at minus 0 5 instead of zero corrects emp ically for th s minute elasticity error

b If the concentrat on of absorbable gas in the sample is high larger than usual amounts of absorbent must be used. The CO absorbent will absorb 10 times its volume of CO w thout significantly alter ng vapor tension and the O ab so bent will absorb 5 to 6 times its own volume of O before turn ng brown and giving poor results

c Carefully avoid sucking the rinsing solution down through S 2 The rinsing solution will gradually remove the grease coating allowing the trapped traces of acid to move in the stopcock This is usually first detected by the fact that the micrometer misses the zero after the end of the analysis To check the tightness of S 2, fill B with the acid solution and adjust the gas meniscus to the mark With S 2 in Position I hang the leveling bulb low on the ring or handle J If S 2 leaks the meniscus will move slowly down If the bulb is hung high the meniscus will rise Unless the meniscus stays at the mark, dry and regrease S 2 and tie the leveling bulb to the ring with a piece of string so that it cannot be lowered below the glass joint

SECTION VI

PHYSIOLOGICAL MEASUREMENTS (Continued)

D RESPIRATORY METABOLISM

1 Collection of Expired Air—The Tissot Gasometer

Reference

Bock A. V Dill D B and Talbott J H Studies in Muscular Activity I Determination of the Rate of Circulation of Blood in Man at Work J Physiol 61 121 13 (Oct) 19 8

Principle

The subject s nose is clamped and through a mouthp ece he passes all his expired air into a calibrated counterpoised metal cylinder which is seated by means of a water seal in a second cylinder and wh ch is free to move with respiration From the calibration constant and the time the pulmonary ventilation may be calculated.

Apparatus

1 Tissot gasometer of 100 or 250 liter capacity (see Fig 43) of use during walking The nner bell must be counterpoised accurately so as not to impede respirat on The gasometer should be calibrated against a standard zed wet gasometer
2 One mouthp ece nose clip assembly Depending on whether the subject is to exer cise v olently or not the valves should be flutter valves or butterfly valves For all exerc se flutter valves are required From the expiratory valve corrugated 1 to nch gas mask tubing leads to the intake of the Tissot gasometer One comfo table screw type nose clip is nee led
3 One barometer either mercury or aneroid sensitive to 0 5 mm Hg
4 One watch with a second hand p eferably a stopwatch
5 Gas sampling tubes Barcroft des gn in racks of four filled with mercury
6 A three way large bore aluminum stopcock inserted between the ex t valve of the mouthpiece assembly and the gasometer Two posit ons are required (a) subject to gasometer (b) subject to outside

Reagents

1 Alcohol 80% fo ste li ng the mouthp ece after use

Procedure

1 Check the gasometer for counterpoise and for water level which should be 1 inch below the rim of the outer cyl nder If more water is needed be sure that its tempe ature is close to room temperatu e Check that the hanging scale is not scraping aga nst the s des of the guides
2 Befo e g ving the mouthpiece to the subject wash t in 80% alcohol and dry it
3 Check leakage of valves by blowing through them and check the r tightness of fit by blowing backward through them There should be no leakage
4 Place the nose cl p fi mly on the subject s nose and have h m test for leaks past the cl p by trying to blow air through h s no e
5 Be sure that the three way tap connects subject to outside air
6 Start the subject performing the task whose energy requirement s to be measured
7 Turn three way tap to connect the subject with the Tissot gasometer



Fig 43 —Use of Ti sot gasometer for collecting specimens of expired air (Reproduced by courtesy of Dr H. S Belding)

8 Allow bell to fill about one third full with air and turn the tap to connect the subject with air

9 Remove rubber stopper from thermometer hole and by manual pressure expel all air from the gasometer

10 Repeat steps 7 8 and 9 twice more This insures that there will be no mixing error from dead space air already in the gasometer

11 Now read the gasometer scale (Reading 1) to the nearest mm

1 Start stopwatch and turn tap to conne t subject with gasometer

13 At exactly 1 minute and every minute desired thereafter read the scale This is a check on the consistency of the subject and apparatus

14 At a desired time usually 5 minutes for low rates of pulmonary ventilation the tap is turned to connect subject with outside air and the final scale reading is made (Reading 2)

15 Turn on mixing fan and read the temperature in the bell to the nearest 0 1 C

16 Read the baromet r to the nearest 0 5 mm

17 Take specimens of the contents of the bell from the thermometer hole into Barcroft gas sampling tubes making sure that
   a The contents of the bell have been mixed and
   b The Barcroft gas sampling tube is flushed three times in the following order contents of sampling tube to outside air contents of bell into sampling tube contents of sampling tube to outside air

Calculation

Pulmonary Ventilation =

$$\frac{(\text{Reading 2} - \text{Reading 1})}{(\text{minutes})} \times \text{T ssot facto} \times \text{STP factor}$$

Where

Pulmonary ventilation is expressed in liters of gas expired per minute reduced to 0 C and 60 mm Hg Readings ° and 1 are the last and first readings expressed in cm to the nearest mm Tissot factor is the liters of gas equivalent to a rise of 1 cm in the Tissot bell and STP factor is the factor fo reducing any volume of wet gas at any temperature and pressu e to 0 C and 760 mm Hg dry (see Fig 45 in section on metabolic calculations)

Example

A subject was lying quietly

Reading 1 was 5 1 cm Reading was 60 5 cm T was 4 C barometer was 750 mm and the time was 5 minutes The Tissot factor for this particular gasometer was 1 3 l/cm. (Note that every Tissot has its own calibration figure to be deter mined at purchase)

Pulmonary vent lation l/min dry STP =

$$\frac{(60.5 - 5.1)}{5} \times 1\,{}^{\circ}3 \times 0.89 = 12.1 \text{ l/min}$$

Precautions

1 The chief sources of error in th s method are
   a Leakage around nose clip or in valves
   b Inaccurate read ng of Tissot scale
   c Dilution of contents of bell with outside ai which will not vitiate calcula lation of pulmonary ventilation but which will destroy the validity of all metabolic calculations
   d Inaccurate use of Barcroft gas sampling tubes

2 If room a r is significantly different in composit on from outdoor air it may be necessary to pipe outdoor air into the intake valve of the subject s valve as sembly All metabolic calculations assume outdoor air composition of the insp red air

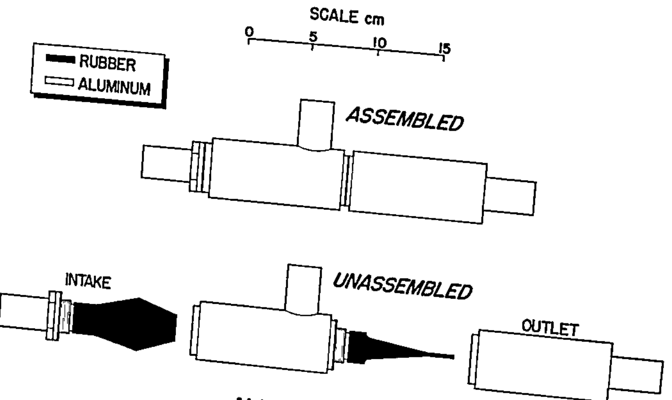


Fig 44 —Valve ass mbly for c ll ction of expired ai wh n ate of pulmonary v ntilation a moderate.

2 Collection of Expired Air—The Douglas Bag Technique

Reference

Haldane J S and Priestley J G Respiration Oxford 1935 Clarendon Press

Principle

The subj ct s nose is clamped and through a mouthpiece he passes all of his expired air into an air tight ba Two one way valves allow him to breathe in only outside air and to expire only into the bag The total pulmonary ventilation in a given time is measured by pas ing the contents of the bag th ough a gas meter and samples are taken for analys s

Apparatus

1 One barometer either mercury or aneroid sensitive to 0 5 mm Hg
2 One watch with a second hand preferably a stopwatch
3 One mouthpiece assembly (Fig 44) This should be usable even when the subject is exerc sing violently A rubber mouthp ece s mounted in the expiratory hole of a gas mask of which the goggles are removed and the nose is cut out The insp ratory cann ster is removed and the head straps left intact The rubber mouthpiece is attached to a pa r of aluminum 1 inch tubes containing rubber flutter valves one in piratory the othe exp ratory which lie snugly along the outside of the face of the mask From the exp ratory valve a piece of corrugated 1 inch gas mask tubing leads o e the head of the subject to the bag assembly described below
4 One comfortable nose cl p screw type
5 *One Douglas bag assembly This consists of one 1°5 1 Douglas bag (Davol* Rubber Company Prov dence Rhode Island) and a light pack board for the subject to wear on h s back The bag consists of two layers of canvas w th rubber sheeting cemented between and must be absolutely leak proof when the exit tube is clamped off The upper co ne s of the bag can be hooked to the upper corners of the pack board. The ex t tube of the bag should then reach the level of the subject s neck A strong flat brass clamp should be placed on the exit tube of the bag wh ch is then attached to a 1 inch way aluminum tap One l mb of the tap is attached to the bag and one to the gas mask tubing from the subject s expiratory valve The third l mb is to outside air The th ee way tap should allow either of two circuits for the subject s exp red air i e mouth—valve—gas mask tub ng— way tap—and thence either (a) outside air or (b) nto Douglas bag
6 One dry gas meter of at least ° 5 cu. ft per minute capacity (Tufts Meter Company Albany N Y) A the mometer should be mounted in the intake side of the gasmeter
7 Rack of gas sampling tubes Orthodox mercury filled tubes must be avoided in the field when temperatures are extreme A satisfactory sampl ng tube consists of a 50 ml syringe oiled w th heavy oil and fitted with a inch length of pressure tubing over the nozzle The tubing is clamped off with a screw clamp The 3 way alum num tap must have a s de arm fo taking gas samples This is closed with a rubber nipple all the t me except when samples are taken

Reagents

1 Heavy oil for the sampl ng syringes
80% alcohol to steril e the mouthp ece after use

Procedure

1 Check the Douglas bag for leaks the night before us ng it Th s is done by hooking the bag to the outlet of the gas meter runn ng in a measured volume of air clamping off the neck of the b g placing 0 lbs or more on the bag for an

hour and running the contents back through the gasmeter The readings should be nearly the same If there are leaks they can be patched temporarily with ordinary tire patching

2 Before asking the subject to take the mouthpiece wash it with 80% alcohol and dry it

3 Place the nose clip firmly on the subject s nose Have him test for leaks past the clip by trying to blow air through his nose This step should never be omitted.

4 Place the mouthpiece assembly on the subject s head fit the mouthpiece so that it is comfortable and tie the headstraps snugly

5 Empty the Douglas bag completely of air by laying it flat on the ground with the exit open rolling it up snugly and clamping off the exit tube with the brass clamp

6 Hang the Douglas bag on the pack board connect the exit tube to the 3 way tap and finally connect the corrugated tube from the mouthpiece to the 3 way tap

7 Be sure that the 3 way tap is adjusted so that the subject s expired air is going to the outside

8 Start the subject performing the task whose energy requirement is to be measured Let him continue this long enough to ' warm up before Step 9

9 Take off the brass clamp Simultaneously start the watch at 0 and turn the 3 way tap so that all of the expired air goes into the bag

10 Allow the subject to breathe into the bag while continuing his task until the bag seems about 3/4 full During rest this takes 10 minutes during light activity about 5 and during moderate activity about 3 minutes Vigorous activity is difficult to control or measure The bag may fill up in about a minute

11 *Simultaneously note the time to the nearest second and turn the 3 way tap so* that the subject is breathing to the outside He may now stop work

12 Clamp off the neck of the bag disconnect it along with the 3 way tap and remove it from the pack board to the neighborhood of the gas meter

13 Mix the bag s contents by laying it on the floor and kneading it a few times

14 Connect the 3 way tap with bag attached to one limb to the inlet of the gas meter

15 Unscrew the clamp from a sampling syringe and connect the pressure tubing to the sampling nipple on the 3 way tap

16 Take a reading on the dial of the gas meter V

17 Open the 3 way tap so that the contents of the bag flow into the gas meter *not* to the outside air

18 Put steady pressure on the bag and when it is about ⅙ empty take a sample by drawing the syringe full and empty 3 times while the gas is flowing steady through the 3 way tap Finally fill the syringe clamp off the rubber tube and after taking a duplicate sample close the sampling nipple of the 3 way tap

19 Express all of the bag s contents through the gas meter Toward the end lay the bag flat on the ground and roll it up tightly from the bottom

20 Clamp off the neck of the bag and take the final reading on the gas meter V

21 Read the temperature in the intake of the gas meter to the nearest ½ C and read the barometer to the nearest ½ mm Hg

Calculations

$$\text{Pulmonary ventilation} = \frac{(V - V)}{(\text{time})} \times \text{STP factor}$$

Where Pulmonary ventilation is expressed in liters of gas expired per minute reduced to 0 C and 760 mm Hg dry

V *is the first reading of the gas meter* in liters

V is the second reading of the gas meter in liters

STP factor is the factor for reducing gas at any temperature and pressure wet to 0 C and 760 mm Hg dry as shown in Fig 45

If the gas meter reads cubic feet the conversion factor by which readings are multiplied to give liters is 28 3 Time is expressed in *minutes and decimal fractions.*

Example

V 91 5 V 185 3 Temp C Barometer 760 mm

Time of collection 4 minutes 26 seconds = 4 and 26/60 = 4 43 mins

Pulmonary ventilation $= \frac{(185\ 3 - 91\ 5)}{(4\ 43)}$ (0 90) = 19 0 1 *per min* STP

Precautions

1 Test the bag for leaks every few days

Be sure to have the subject test for leaks through the nose or around the mouth piece

3 Timing must coincide with turning the taps.

4 Every time a tap is turned be sure that the gas flow will be in the direction desired

5 Do not forget to read the temperature and barometric pressure every time samples are measured

6 In vigorous activity when a significant oxygen debt is accumulated it is necessary to have the subject exercise for a minute or so then stop and collect expired air while he is resting after work for about 5 minutes If this is not done the estimate of caloric expenditure during work will be erroneously low

3 Respiratory Metabolic Calculations

Reference

Haldane J S and Priestley J G Respiration Oxford 1935 Clarendon Press

Principle

From the pulmonary ventilation and the carbon dioxide oxygen and nitrogen content *of the expired air compared with the inspired a the consumption of oxygen* and production of carbon dioxide are calculated From these data energy expenditure may be *computed*

Pulmonary Ventilation

This is expressed as liters of air expired per minute the volume of gas being reduced to the standard temperature and pressure 0 C and 760 mm Hg dry The formula is

$$\frac{B - W}{760\ (1 + 0\ 00367\ t)}$$

Where B is ambient barometric pressure W is the vapor tension of water mm Hg at the temperature of the gasometer and t is the temperature of the gasometer in C To save computation one uses the line chart which was devised by Dr R C Darling (Fig 45)

Respiratory Quotient (R Q)

This is the ratio of CO *expired divided by* O *consumed i e*

$\frac{CO\ \text{production}}{O\ \text{consumption}}$ and is used in the calculations which follow

True Oxygen, True Carbon Dioxide and R.Q

True Oxygen represents the number of ml of oxygen consumed for every 100 ml of air expired It is based on several considerations as follows One desires to know how much oxygen is removed from the air breathed in but the only measurements made are the volume of air expired and its oxygen carbon dioxide and nitrogen contents The volume of inspired air is usually not the same as that of expired air This is because the R Q has to be exactly 1 00 for inspired to equal expired a If the R Q is less than one as is usually the case in rest or moderate

LINE CHART FOR DETERMINING FACTORS TO REDUCE SATURATED GAS VOLUMES TO DRY VOLUMES AT 0°C AND 760 MM HG

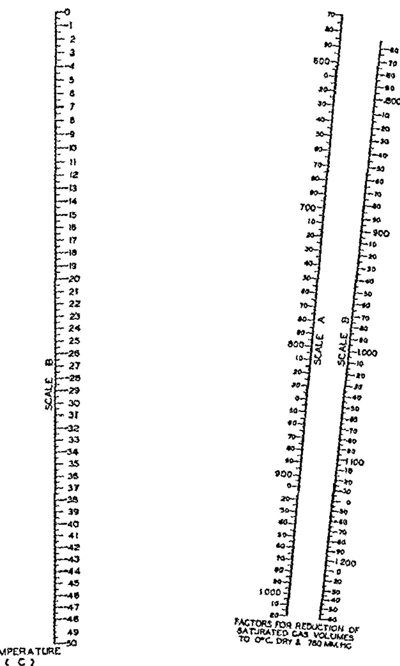


Fig 45—Line chart for converting wet gas volumes to STP and dry as drawn by Dr R. C. Darling

exercise then the oxygen removed from inspired air is only partly replaced by carbon dioxide and if one l has been inspired less than one l will be expired The nitrogen concentration in this case will be higher in expired than in inspired air One knows the nitrogen concentration in outdoor air to be 79.04% and the concentration in expired air is determined by Haldane analysis The volume of inspired air may be calculated from the expired air by the formula

$$\text{Vol inspired} = \text{Vol expired} \times \frac{\%\text{N in expired air}}{79.04} \qquad \text{(Equation 1)}$$

The total volume of oxygen breathed in (*not* all consumed) is then

$$\text{Vol O inspired} = \text{Vol air inspired} \times \frac{\%\text{O of inspired air}}{100} \qquad \text{(Equation)}$$

The percentage of oxygen in practically all outdoor air is 20.93 Hence

$$\text{Vol O inspired} = \text{Vol air inspired} \quad \frac{20.93}{100} \qquad \text{(Equation 3)}$$

The volume of oxygen expired (amount not consumed) is

$$\text{Vol O expired} = \frac{\%\text{O in expired air}}{100} \quad \text{vol air expired} \qquad \text{(Equation 4)}$$

The amount of oxygen consumed is

$$\text{O consumed} = \text{Vol O inspired} - \text{Vol O expired} \qquad \text{(Equation 5)}$$

Substituting values from equations (3) and (4)

$$\text{O consumed} = \text{Vol air inspired} \times \frac{20.93}{100} - \text{Vol air expired} \times \frac{\%\text{O in expired air}}{100}$$

Substituting values from equation (1)

$$\text{O consumed} = \text{Vol air expired} \times \frac{\%\text{N in expired air}}{79.04} \times \frac{20.93}{100} - \text{Vol air expired} \times \frac{\%\text{O in expired air}}{100}$$

Simplifying

$$\text{Vol O consumed} = \frac{\text{Vol air expired}}{100} (\%\text{N in expired air} \times 0.265 - \%\text{O in expired air}) \qquad \text{(Equation 6)}$$

The factor (%N in expired air × 0.265 − %O in expired air) is the True Oxygen the number by which the volume of expired air (divided by 100) is multiplied to give the oxygen consumption

By steps similar to the derivation of oxygen consumption the carbon dioxide expired becomes

$$\text{Vol CO expired} = \frac{\text{Vol air expired}}{100} \left(\%\text{CO in expired air} - \frac{\%\text{N in expired air}}{79.04} \times \%\text{CO in inspired air}\right)$$

In the case of ordinary outdoor air this becomes

$$\text{Vol CO expired} = \frac{\text{Vol air expired}}{100} (\%\text{CO in expired air} - 0.03) \qquad \text{(Equation 7)}$$

The factor (%CO in expired air − 0.03) is the True Carbon Dioxide the number *by which the volume of expired air (divided by 100) is multiplied to give the carbon dioxide production*

The respiratory quotient R Q is obtained by dividing equation (7) by equation (6) and is

$$\text{R Q} = \frac{(\%\text{CO in expired air} - 0.03)}{(\%\text{N in expired air} \times 0.265 - \%\text{O in expired air})} \qquad \text{(Equation 8)}$$

To facilitate the computation of True O and R Q a line chart has been constructed A string stretched between the % O_2 and % CO_2 in expired air passes across lines giving R Q and True O ' directly (See Fig 66 at end of this book)

Oxygen Consumption and CO Production

The oxygen consumption in liters per minute is

$$O_2 \text{ l/min} = \frac{\text{Pulmonary ventilation l/min}}{100} \times \text{True O} \qquad \text{(Equation 9)}$$

The carbon dioxide production in liters per minute is

$$CO \text{ l/min} = \frac{\text{Pulmonary ventilation l/m}}{100} \times \text{True CO} \qquad \text{(Equation 10)}$$

Energy Expenditure

It is conventional to expre s the metabolic cost of a given task in terms of Calories per hour The Calorie is the amount of heat necessary to raise the temperature of 1 kilogram of water 1 C and is the basic unit of all nutritional energy calculations To compute the cost of a given task the formula is used

Cals/Hr = (O consumption l/min) × *60* × 5 0 = O l/min × *300*

The factor 5 0 is an average figure representing the Caloric equivalent of 1 liter of oxygen consumed during moderate activity

Since most of the common forms of work require an energy expenditure which is directly proportional to the body weight most tables of metabolic costs of activity are expressed on the basis of a standard man weighing 150 lbs or 70 kg To compensate for variations in weight energy expenditure in work may be *expressed* in terms of Cals /hour and kg body weight or converted to a standard body weight as follows

$$\text{Cals /Hr (standard man)} = \text{O l/min} \times 300 \times \frac{150}{\text{body weight lbs}}$$

For some special purposes energy expenditure is computed on the basis of body *area* not body *weight* This will be discussed in the section on basal metabolism

Example of Metabolic Calculations for a Walking Man

Company marched 3 5 miles in 50 mins on the level
Weight of subject stripped 145 lbs
Weight of clothes helmet pack rifle canteen 29 lbs
Weight of metabolic apparatus 13 lbs

A. Douglas Bag Calculations
Collection started 7 hr 30 0 ended 7 hr 3? 0 = ? 00 mins
Gas meter readings—Start 448 0 L End 66? 9 1 = ?14 9 1
Temperature 21 9 C Barometer ?57 mm
STP factor (from Fig 45) = 0 880

$$\text{Pulmonary ventilation l/min STP dry} = \frac{(214\ 9)}{(?\ 00)} \times 0\ 880 = 7\ 0$$

B Haldane Analysis
% CO_2 = 3 81 % O = 16 80

C Calculation of O consumption
From line chart for True O (See Fig 66 in back)
R Q = 0 89 True O = 4.23

$$\text{O consumption l/min} = \frac{\text{Pulmonary Vent}}{100} \times \text{True O} = \frac{27\ 0}{100} \times 4\ ?3 = 1\ 14$$

$$\text{CO production l/min} = \frac{\text{Pulmonary Vent}}{100} \times \text{True CO} = \frac{27\ 0}{100} \times (3\ 81 - 0\ 03) = 1\ 0$$

D Calculation of energy expenditure

Cals/min = O l/min × 5
= 1 14 × 5 = 5 7

Cals/50 min = 5 7 × 50 = 285

Cals for 10 min rest assumed to be °0

Energy expended in one hour that consists of 3 miles marching in 50 minutes and 10 minutes rest = 285 + 0 = 305 Cals

Protein Metabolism During Work

The above calculations and examples n glect two factors which are not important in field measurements b t are theo etically important Some of the Calories are derived from protein and one liter of oxygen consumed represents different amounts of heat depending on the ratio of fat carbohydrate and protein being consumed by the body at the time of measurement P otein metabolism is determined from the urinary nitrogen excretion and the non protein R Q is determined from the over all R Q (obtained as described above) and the protein metabolism Fo details of this type of calculation see Peters and Van Slyke Quantitative Clinical Chemistry pp 05 15 Vol II Baltimore 193 Williams and Wilkins

4 Basal Metabolic Rate

Reference

Du Bois E F Basal Metabolism in Health and D sease ed 3 Philadelphia 1937 Lea and Febiger

Principle

The basal metabolic rate (BMR) is the energy expenditure of a subject resting quietly after at least 8 ho s sleep and at least 1 hours after the last meal When expressed on the basis of Calorie production per square meter of body area it is remarkably constant for healthy people of the same age and sex

Apparatus and Reagents

Same as under measurement of expired air In add tion an oral clinical ther mometer a clinical weighing scales and a measu ing stick are needed

Procedure

1 For an accurate test the subject must have had at least 8 hours sleep and no food for at least 1 hours T is also includes alcoholic beverages coffee tea and tobacco He lies down comfortably for at least ½ hour in a quiet place before the test

2 Metabolism is determined while he is still resting as described in the section on expired air Count the respirations for 1 minute during the process If he is too excited the test will be invalid

3 B fore he gets up and immediately after the mask and mouthpiece are removed measure and reco d the pulse and o al temperature This is necessary to insure that one is not deal ng with an infection Fever invalidates the test

4 Measure the subject s height in stocking feet and we ght with a minimum of clothing

5 Record h s age to the nearest year

Calculation

1 Calculate oxygen consumption and R Q as described previously If the R Q is outside the range 0 80 – 0 90 the test s nvalid

From his he ght and weight determine the subject s surface area from the line chart (Fig 46) This nomogram was computed from the DuBois Meeh formula for surface area

SURFACE AREA FROM HEIGHT AND WEIGHT

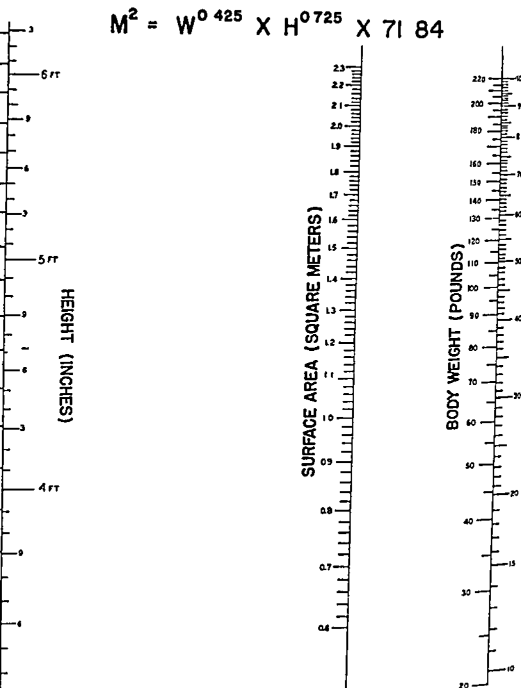


Fig 46 —Nomogram constructed from the Du Bois Meek formula for surface area.

HEAT PRODUCTION (Cal/M²/hr) FROM SURFACE AREA AND OXYGEN CONSUMPTION (R Q 0 85)

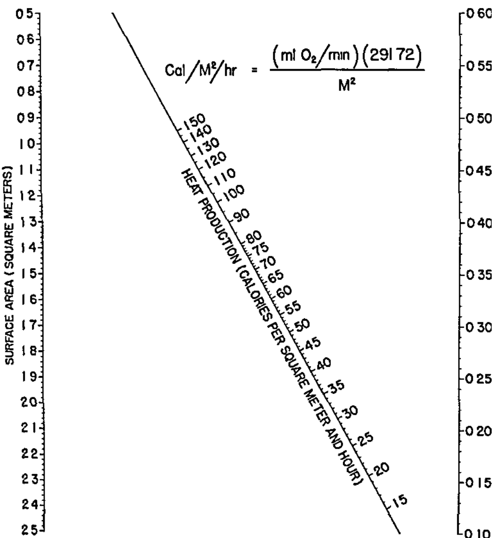


Fig 47 —N m gram f computing heat p o luction

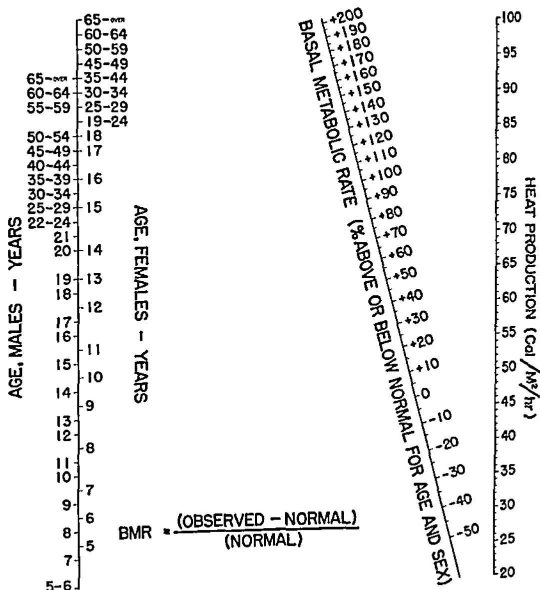


Fig 48 —Nomogram for computing BMR from heat production, age, and sex. The Boothby Sandiford standards were employed.

3 From oxygen consumption and surface area compute heat production (Cals per square meter per hour) The line chart in Fig 47 was drawn to facilitate this computation.

4 Complete the subject s basal metabolic rate (i.e % above or below normal) by the formula

$$BMR = 100 \times \frac{(\text{Observed Cals /m}^2\text{/hr} - \text{Average normal})}{\text{Average normal}}$$

(Where average normal refers to the Boothby Sandiford values)

5 The BMR line chart in Fig 48 facilitates the above calculation It was computed from the Boothby Sandiford normal values

Example

Subject R D Sex Male
Age ?6 years
Ht 5 ft 7½ inches
Wt 19 lbs
Pulse rate 69
Temp 98 4 F
Pulmonary Ventilation l/m n STP 8 23
Expired air % CO 3 5? % O 17 08
RQ 0 89 True O 3 95
Oxygen Consumption l/min 0 3 5
Surface Area ? 11 sq m. (Fig 46)
Heat production 44 5 Cals /m /hr (Fig 47)
BMR + 12 (Fig 48)

Precautions

1 The commonest source of error are
a The subject is not truly basal.
b The subject is not comfortable and at ease
2 The above calculations and examples neglect the two factors protein metabolism and the varying heat equivalent of one liter of oxygen depending upon the ratio of protein fat and carbohydrate being consumed by the body These factors are not important for field use but are theoretically important The matter is discussed at the end of Metabolic Calculations
3 If the pulse rate is above 80 or the resp rations above 25 the subject is either not basal or else has some patholog cal condition If the temperature is elevated he is either not basal or else has some disease (Exercise of moderate severity elevates the body temperature)

5 *Metabolism During Work—The Treadmill*

Reference

Robinson S Experimental Studies of Physical Fitness in Relation to Age Arbeits physiologie 10 ?51 3 3 (No 3) 1938

Principle

The motor driven treadmill is the best apparatus yet devised for general studies of the response of man to exercise A typical protocol is described for studying respiratory and cardiovascular responses during moderate exercise (walking) and exhausting exercise (*running*)

Apparatus

1 The treadmill (see photograph Fig 49) The treadmill has a motor driven end less leather belt that can be operated at any speed up to 20 miles per hour The front end can be raised up to make a grade of 20% It is possible to make the

work so easy that an ordinary individual can walk for hours and so severe that the best runners can be exhausted in a very short time (seconds) (Purchased at A R Young Co, Indianapolis Indiana)

2 The stationary sides of the treadmill carry a superstructure on which are suspended the rubber hose connection (1 inch inside diameter), and a two way flutter valve system. The hose connection brings fresh air from the outside to the valve and empties the expired air into the gasometer

3 The gasometer is a 500 liter copper bell type that is immersed in water Each side of the bell is weighed down so that it balances perfectly when all the outlets are open The measuring scale is in centimeters Installed at the top of the bell is an electric stirrer for mixing the gas before sampling At the inlet of the gasometer is a baffle mixer of 10 liter capacity from which samples of gas may be drawn while the subject is working, without interfering with the total collection in the gasometer

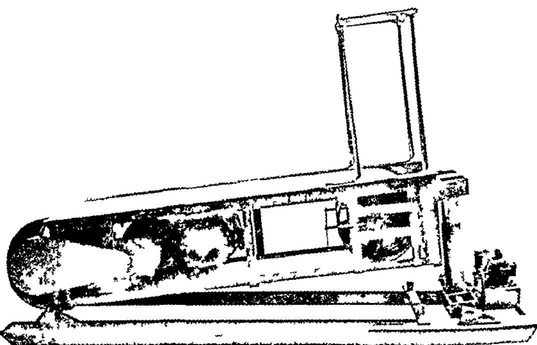


Fig 49—Electrically driven treadmill manufactured by A. R. Young Indianapolis Indiana. (Photograph by courtesy of A R Young C mpany Indianapolis Ind)

4 An accurate sweep second clock and a stopwatch

5 Spare Douglas bags that have a capacity of ?00 liters

6 Rack of gas sampling tubes (Barcroft type) with an approximate capacity of 30 ml each

7 A cardiotachometer for recording pulse rates with electrodes and electrode paste The electrodes are able to pick up electrical currents that are generated at each contraction of the heart The current is then amplified to operate a relay and a pen on a moving tape thus yielding a record of the heart rate as work is going on The three electrodes are fastened on two lengths of elastic tape The first electrode is placed just to the left of the sternum at the level of the second rib

The second electrode is placed over the apex of the heart and the third or ground which is on the same elast c tape as the second is on the right side

8 Micro pipettes for lactic acid and blood sugar of 0 5 ml and 0 1 ml capacity
9 Watch glasses for collecting the blood during exercise
10 A lan et for pricking finger to get blood.
11 Centrifuge tubes 15 ml and 50 ml round bottom with rubber stoppers
1 Barometer
13 A Haldane gas analysis apparatus

Reagents

1 Clean mercury for gas sampling tubes
Heparin solution (1 drop dried on a watch glass)
3 Dilute acid tungstate for blood sugar 20 ml of 10% sodium tungstate solution 0 ml of ⅔ N sulfuric ac d ($H SO$) Dilute to 1000 ml w th water
4 Distilled water for lact c acid tubes

Procedure—Moderate Exercise

1 The subject dressed in gymnasium clothes is connected to the cardiotachometer with the three electrodes fastened around h s chest
- The subject s ts quietly at the side of the t eadmill while h s re ting pulse is recorded and his blood p e sure is determ ned
Subject straddles the treadm ll while the treadmill is started (3 5 m p h on 8 6% grade)
4. Subject sta ts walking on s gnal from expe menter Notation on heart rate tape is made
5 After app ox matelv five m nutes walking the subject applies mouthpiece and nose cl p and the valve is turned so that exp red air enters the gasometer
6 In the course of the next fou m nutes the gasometer is washed out with three portions of exp red air and finally a gasometer reading is taken after expulsion of the last wash portion
7 At the ninth minute of the walk collection of exp red air is started and contin ued for five minutes the gasometer read ngs being noted every m nute
8 At approximately ten m nutes of the walk finger blood s taken for sugar and lactate
9 After a total of 15 minutes walk the subject steps off the treadmill s ts down and rests for ten minutes during which t me the following procedures are car ried out
   a Duplicate gas samples taken from gasometer and temperature noted Change gas circuit to mix ng chamber
   b Subject s pulse rate and blood p essure recorded at frequent intervals

Procedure—Exhausting Exercise

1 The usual grade for exhausting work is the same as for the walk (8 6%) but with the speed of the treadmill increased to 7 0 m p h Ord narily the subject is not requ red to run longer than five minutes To obtain exhaustion with n five min utes in well trained subjects the grade may need to be increased If the grade is to be changed it is changed during the half minute before the run starts but not until the exp red air and blood have been sampled as above
- In t al gasometer read ng s taken aga n
3 Subject prepares for run straddles treadmill holding the s des and is instructed to signal w th h s hand if he des res to stop before five minutes
4 The treadmill is started at 7 0 m.p h. and on s gnal the following procedures oc cur simultaneously
   a The subject starts running
   b Valve is turned into gasometer
   c Cardiotachometer is marked

5 Gasometer readings are taken every half minute during run
6 Expired air samples are taken over an entire minute for the second, third fourth and fifth minutes (the first minute is not of sufficient value), or for as long as the subject runs Samples of less than 20 *seconds are of doubtful value*

Procedure—Recovery After Exhausting Exercise

1 When subject stops running by jumping to a straddling position (keeping the mouthpiece in and the nose clip on) the *following must be done precisely at the* time he stops running
   a Valve at treadmill superstructure is turned to collect expired air in Douglas bags for three minutes
   b Cardiotachometer tape is marked
   c Time is noted
   d Treadmill is stopped Subject sits on tall stool in place on treadmill still breathing through mouthpiece
2 As soon as possible the following additional steps should be taken
   a Final gasometer reading is taken
   b Temperature of expired air in gasometer is noted
   c Gasometer is emptied Connection removed from mixing chamber and placed on direct connection to gasometer Valve turned for collection and reading noted
3 Blood pressure is taken as soon as possible preferably during the first minute after subject stops running
4 At the end of three minutes valve at treadmill superstructure is turned to gasometer
5 Gasometer readings are taken every minute during the remainder of the 15 minutes
6 Finger blood samples for blood sugar and lactate are taken at 5 10 and 15 minutes during recovery
7 Cardiotachometer is left running at least for the *first five minutes of recovery* and may be turned off then and used only to take heart rate during 10th and 15th minutes of recovery
8 At 15 minutes gasometer valve is closed temperature is recorded and final reading *is noted*
9 Two expired air samples are taken from gasometer (3 15 minutes of recovery)
10 After gasometer is emptied valves are turned connecting Douglas bag to gasometer and initial reading is noted Two samples of contents of bag are drawn from small side tube on bag as gas is forced to *large gasometer for measurement* of volume Bag is finally rolled to expel all air, connection clamped and gasometer volume and temperature are noted (Three minutes of recovery)
11 Barometric pressure is recorded

(The work experiment as carried out above required at least three *and preferably* four men to guarantee smooth operation)

Calculations and Data Obtained from Work Experiments

1 Blood pressure at rest (*sitting*) *and immediately following exhausting* run
2 Continuous heart rate record counted to obtain following
   a Rest (sitting)
   b Maximum during walk (sixth minute)
   c Maximum during run (usually last minute)
   d First five minutes of recovery May be counted by six second intervals for first minute of recovery and longer intervals up to five minutes
   e At 10 minutes during recovery
   f At 15 minutes during recovery

3 Blood sugar and blood lactate in mg per 100 ml.
   a. During walk
   b At 5 10 and 15 minutes during recovery
4 Metabolism during walk run and recovery are calculated as described in previous sections
5 Oxygen debt This measurement is the difference in oxygen consumption for fifteen minutes following the run and the basal requirement for that period To be complete the period should be 60 minutes

Example of a Protocol of a Typical Experiment

SUBJECT B G ade 8 6%	DATE 8/3/49	BAR PRESSURE 761 mm Hg GASOMETER TEMP 34 C			TISSOT FACTOR 5 × cm STP FACTOR 0 898 COMBINED FACTOR 4 68			
WALK 3 5 m p h	SPIROMETER READINGS cm	DIFF cm	PULMONARY VENTIL L MIN	CO_2 %	O_2 %	R Q	TRUE O_2 %	O_2 USED L/MIN
4 mins	4 6							
5	13 1	8 5	39	4 59	16 8	0 97	4 0	1 8
6	1 0	9	36 9	4 1	15 3	0 91	5 1	1 91
Run 7 0 m p h								
0 mins	4 5							
1	16 9							
1½	5 6							
	34 0	1 1	80 0	4 0	16 48	1 06	4 40	3 5
2½	44 0							
3	53 4	19 4	90 8	4 9	16 6	1 14	4 16	3 78
3½	63 1							
4	3 1	19	9	4 89	16 6	1 17	4 17	3 84
4½	83 0							
5	8 5	19 4	90 8	4 97	16 61	1 19	4 17	3 79
Recovery								
Bag								
0 min	4							
3	44 5	39 8	6 0	4 01	18 09	1 56	2 [illegible]	1 58
Gasometer								
3 mins	4 8							
5	16 5							
7	24 7							
9	3 5							
11	38 8							
13	45 8							
15	51 6	46 8	18 3	3 53	1 9	1 06	3 30	0 60
					Basal O_2 consumption		=	0 30

Net O_2 debt in 15 minutes
3 × (1 58 − 0 30) + 1 × (0 60 0 30) = 7 48 liters

Precautions

1 The whole method depends on good sampling so *be very careful* Rinse out at least 3 times before drawing the final sample
   Accurate timing is almost as important as accurate sampling
2 *It will be noted that the two values for the walk should* and do *check quite closely* and similarly for the last three minutes of the run Generally a man must run at least two minutes at this grade of work before the maximum is obtained
4 We have found that the oxygen debt for 15 minutes is only roughly proportional to the total oxygen debt For precise measurement the period should be 60 minutes or until the O_2 consumption approaches the basal level

5 Gasometer readings are taken every half minute during run
6 Expired air samples are taken over an entire minute for the second third fourth and fifth minutes (the first minute is not of sufficient value), or for as long as the subject runs Samples of less than 20 seconds are of doubtful value

Procedure—Recovery After Exhausting Exercise

1 When subject stops running by jumping to a straddling position (keeping the mouthpiece in and the nose clip on) the following must be done precisely at the time he stops running
   a Valve at treadmill superstructure is turned to collect expired air in Douglas bags for three minutes
   b Cardiotachometer tape is marked.
   c Time is noted.
   d. Treadmill is stopped. Subject sits on tall stool in place on treadmill still breathing through mouthpiece
2 As soon as possible the following additional steps should be taken
   a. Final gasometer reading is taken.
   b Temperature of expired air in gasometer is noted
   c Gasometer is emptied. Connection removed from mixing chamber and placed on direct connection to gasometer Valve turned for collection, and reading noted
3 Blood pressure is taken as soon as possible preferably during the first minute after subject stops running
4 At the end of three minutes, valve at treadmill superstructure is turned to gasometer
5 Gasometer readings are taken every minute during the remainder of the 15 minutes.
6 Finger blood samples for blood sugar and lactate are taken at 5 10 and 15 minutes during recovery
7 Cardiotachometer is left running at least for the first five minutes of recovery and may be turned off then and used only to take heart rate during 10th and 15th minutes of recovery
8 At 15 minutes gasometer valve is clo ed temperature is recorded and final reading is noted.
9 Two expired air samples are taken from gasometer (3 15 minutes of recovery)
10 After gasometer is emptied valves are turned connecting Douglas bag to gasometer and initial reading is noted. Two samples of contents of bag are drawn from small side tube on bag as gas is forced to large gasometer for measurement of volume Bag is finally rolled to expel all air connection clamped and gasometer volume and temperature are noted. (Three minutes of recovery)
11 Barometric pressure is recorded.

(The work experiment as carried out above required at least three and preferably four men to guarantee smooth operation.)

Calculations and Data Obtained from Work Experiments

1 Blood pressure at rest (sitting) and immediately following exhausting run.
2 Continuous heart rate record counted to obtain following
   a. Rest (sitting)
   b Maximum during walk (sixth minute)
   c Maximum during run (u uallv last minute)
   d. First five minutes of recovery May be counted by six se ond intervals for first minute of recovery and longer intervals up to five minutes
   e At 10 minutes during recovery
   f At 15 minutes during recovery

3 Blood sugar and blood la tate in mg per 100 ml
  a During walk.
  b At 5 10 and 15 m nutes dur ng recovery
4. Metabol sm du ing walk run and recovery are calculated as descr bed in pre vious sections
5 Oxygen debt Th s m asurement is the d ff rence in ox gen consumpt on for fifteen m nutes fo lowing the run and the basal requ r m nt for that p r od To be complete the per od should be 60 m nutes

Example of a Protocol of a Typical Experiment

SUBJECT B Grade 8 6%	DATE 8 3 49	BAR PRE S RE 761 mm Hg GASOMETER TEMP 23 4 C			TI S JT FACTOR 5 × cm STP FACTOR 0 896 LO B FD FACTOR 4 68			
WALK 3 5 m.p h	SP ROMETER READ NGS cm	D FF cm	PU 1 ONARY VENTIL L MIN	CO %	O	R Q	TRUE O %	O_2 USED L/M N
4 m ns	4 0							
5	13 1	8	30	4 59	16 6	0 97	4 0	1 87
6	1 0	0	3 9	4 1	15 87	0 91	5 17	1.91
Run 7 0 m p h.								
0 m ns	4							
1	16 9							
1½	25 6							
	34 0	17 1	80 0	4 0	16 43	1 06	4 40	3 5
½	44 0							
3	5 4	19 4	93 8	4 9	16 60	1 14	4 16	3 78
3½	63 1							
4	3 1	19	9	4 89	16 6	1 17	4 17	3 84
4½	83 0							
5	8 5	19 4	90 8	4 9	16 61	1 19	4 17	3 9
Recovery								
Bag								
0 m n	4 7							
3	44 5	39 8	6 0	4 04	18 09	1 6	5	1 58
Gasometer								
3 m n	4 8							
5	18 5							
7	24 7							
9	5							
11	39 8							
13	45 8							
1	51 6	46 8	18 3	3 3	1 9	1 06	3 30	0 60
					Basal O con umpt on		—	0 30

Net O d bt in 15 m nut s
3 × (1 8 0 30) + 12 (0 60 0 30) — 4 8 l te s

Precautions

1 The whole method dep nds on good sampl ng so be very careful P rse out at least 3 t mes before dra ing th final sample
  Accurate tim ng s almost as important as accurate sampl ng
3 It will be noted that the two values for the walk should and do check qu te closely and s m larly for the last three m nutes of the run Generally a man must run at least two minutes at th s grade of work b for th maximum is obtain d
4 We have found that the oxygen debt for 15 minutes is only roughly proport onal to the total oxygen debt. For precise m asurement the period should be 60 minutes or unt l the O consumpt on approaches the basal level

5 Gasometer readings are taken every half minute during run
6 Expired air samples are taken over an entire minute for the second third, fourth and fifth minutes (the first minute is not of sufficient value) or for as long as the subject runs Samples of less than 20 seconds are of doubtful value

Procedure—Recovery After Exhausting Exercise

1 When subject stops running by jumping to a straddling position (keeping the mouthpiece in and the nose clip on) the following must be done precisely at the time he stops running
  a Valve at treadmill superstructure is turned to collect expired air in Douglas bags for three minutes
  b Cardiotachometer tape is marked
  c Time is noted
  d Treadmill is stopped Subject sits on tall stool in place on treadmill still breathing through mouthpiece
2 As soon as possible the following additional steps should be taken
  a Final gasometer reading is taken
  b Temperature of expired air in gasometer is noted
  c Gasometer is emptied. Connection removed from mixing chamber and placed on direct connection to gasometer Valve turned for collection and reading noted
3 Blood pressure is taken as soon as possible preferably during the first minute after subje t stops running
4 At the end of three minutes valve at treadmill superstructure is turned to gasometer
5 Gasometer readings are taken every minute during the remainder of the 15 minutes
6 Finger blood samples for blood sugar and lactate are taken at 5 10 and 15 minutes during recovery
7 Cardiotachometer is left running at least for the first five minutes of recovery and may be turned off then and used only to take heart rate during 10th and 15th minutes of recovery
8 At 15 minutes gasometer valve is closed temperature is recorded and final reading is noted
9 Two expired air samples are taken from gasometer (3 15 minutes of recovery)
10 After gasometer is emptied valves are turned connecting Douglas bag to gasometer and initial reading is noted Two samples of contents of bag are drawn from small side tube on bag as gas is forced to large gasometer for measuremen of volume Bag is finally rolled to expel all air connection clamped and gasometer volume and temperature are noted. (Three minutes of recovery)
11 Barometric pressure is recorded

(The work experiment as carried out above required at least three and preferably four men to guarantee smooth operation)

Calculations and Data Obtained from Work Experiments

1. Blood pressure at rest (sitting) and immediately following exhausting run
2 Continuous heart rate record counted to obtain following
  a. Rest (sitting)
  b Maximum during walk (sixth minute)
  c Maximum during run (usually last minute)
  d First five minutes of recovery May be counted by six second intervals for first minute of recovery and longer intervals up to five minutes
  e At 10 minutes during recovery
  f At 15 minutes during recovery

3 Blood sugar and blood lactate in mg per 100 ml.
   a. During walk
   b At 5 10 and 15 minutes during recovery
4 Metabolism during walk run and recovery are calculated as described in previous sections
5 Oxygen debt This measurement is the difference in oxygen consumption for fifteen minutes following the run and the basal requirement for that period To be complete the period should be 60 minutes

Example of a Protocol of a Typical Experiment

SUBJECT B Grade 8 6%	DATE 8/3/4.	BAR. PRESSURE [illegible]1 mm Hg GASOMETER TEMP 3 4 C			TISSOT FACTOR 5 [illegible] × cm STP FACTOR 0 [illegible] COMBINED FACTOR 4 68			
WALK 3 5 m.p h	SPIROMETER READINGS m	[illegible] cm	[PULMONARY] VENTIL L/MIN	CO_2 %	O_2 %	R Q	TP E [illegible]	O_2 USED L/MIN
4 mins	4 6							
5	13 1	8 5	9 ,	4 9	16 6	0 9,	4 0	1 87
6	[illegible]1 0	9	30 9	4 1	15 97	0 91	5 1	1 91
[Run] 7 0 m p h.								
0 mins.	4 5							
1	16 9							
1½	5 6							
[illegible]	34 0	1 1	80 0	4 0	16 48	1 06	4 40	3 ..
2½	44 0							
3	3 4	19 4	90 8	4 ,9	16 6.	1 14	4 16	3 78
3½	63 1							
4	73 1	19	9	4 80	16 6	1 17	4 1,	3 84
4½	83 0							
5	8 5	19 4	90 8	4 97	16 61	1 19	4 1	3 9
Recovery								
Bag								
0 min	4 7							
3	44.5	39 8	t 0	4 01	18 09	1 r	? 5	[illegible]
Gasometer								
3 mins	4 8							
5	16 5							
7	[illegible]4 7							
9	[illegible]5							
11	39 8							
1[illegible]	4. 8							
15	51 6	46 8	18 3	3 53	1 59	1 06	3 0	0 60
					Basal O_2 consumption =			0 30

Net O_2 debt in 15 minutes
3 × (1 58 − 0 [illegible]0) + 1[illegible] × (0 60 − 0 30) = 7 48 liters

Precautions

1 *The whole method depends on good sampling so be very careful* *Rinse out at least 3* times before drawing the final sample.
2 Accurate timing is almost as important as accurate sampling
3 It will be noted that the two values for the walk should and do check quite closely and similarly for the last three minutes of the run Generally a man must run at least two minutes at this grade of work before the maximum is obtained
4 We have found that the oxygen debt for 15 minutes is only roughly proportional to the total oxygen debt. For precise measurement the period should be 60 minutes or until the O_2 consumption approaches the basal level

5 Gasometer readings are taken every half minute during run
6 Expired air samples are taken over an entire minute for the second, third fourth and fifth minutes (the first minute is not of sufficient value) or for as long as the subject runs Samples of less than 20 seconds are of doubtful value

Procedure—Recovery After Exhausting Exercise

1 When subject stops running by jumping to a straddling position (keeping the mouthpiece in and the nose clip on), the following must be done precisely at the time he stops running
   a Valve at treadmill superstructure is turned to collect expired air in Douglas bags for three minutes
   b Cardiotachometer tape is marked
   c Time is noted
   d. Treadmill is stopped. Subject sits on tall stool in place on treadmill still breathing through mouthpiece
2 As soon as possible the following additional steps should be taken
   a Final gasometer reading is taken
   b Temperature of expired air in gasometer is noted
   c Gasometer is emptied Connection removed from mixing chamber and placed on direct connection to gasometer Valve turned for collection, and reading noted
3 Blood pressure is taken as soon as possible preferably during the first minute after subject stops running
4 At the end of three minutes valve at treadmill superstructure is turned to gasometer
5 Gasometer readings are taken every minute during the remainder of the 15 minutes
6 Finger blood samples for blood sugar and lactate are taken at 5, 10 and 15 minutes during recovery
7 Cardiotachometer is left running at least for the first five minutes of recovery and may be turned off then and used only to take heart rate during 10th and 15th minutes of recovery
8 At 15 minutes gasometer valve is closed temperature is recorded and final reading is noted
9 Two expired air samples are taken from gasometer (3 15 minutes of recovery)
10 After gasometer is emptied valves are turned connecting Douglas bag to gasometer and initial reading is noted Two samples of contents of bag are drawn from small side tube on bag as gas is forced to large gasometer for measurement of volume Bag is finally rolled to expel all air connection clamped and gasometer volume and temperature are noted. (Three minutes of recovery)
11 Barometric pressure is recorded

(The work experiment as carried out above required at least three and preferably four men to guarantee smooth operation)

Calculations and Data Obtained from Work Experiments

1 Blood pressure at rest (sitting) and immediately following exhausting run
2 Continuous heart rate record counted to obtain following
   a Rest (sitting)
   b Maximum during walk (sixth minute)
   c Maximum during run (usually last minute)
   d First five minutes of recovery May be counted by six second intervals for first minute of recovery and longer intervals up to five minutes
   e At 10 minutes during recovery
   f At 15 minutes during recovery

3 Blood sugar and blood lactate in mg per 100 ml
   a *During walk*
   b *At 5 10 and 15 minu es dur ng recove y*
4 Metabolism during walk run and recovery are calculated as described in previous sections
5 Oxygen debt Th s measurement is the difference in oxyg n con umption for fifteen minutes following the run and the basal requirement for that period To be complete the period should be 60 m nutes

Example of a Protocol of a Typical Experiment

SUBJECT B Gra le 8 6%	DATE 8/3/49	BAR PRESSURE 761 mm Hg GASOMETER TEMP 34 C			TISSOT FACTOR 5 2 × cm STP FACTO 0 896 COMBINED FACTOR 4 68			
WALK 3 5 m.p h	SPIRO IETER REAI INGS cm	DIFF cm	PULMONARY VENT L MIN	CO_2 %	O_2	R Q	TRUE O_2 %	O_2 USED l/MIN
4 mins	4 6							
5	13 1	8	39 7	4 59	16 6	0 97	4 0	1 97
6	21 0	7 9	36 9	4 /1	15 97	0 91	5 17	1 91
Run 7 0 m p h.								
0 m ns.	4 5							
1	16 9							
1½	26							
	34 0	1 1	80 0	4 0	16 48	1 06	4 40	3 5
½	44 0							
3	53 4	19 4	91 8	4 3	16 65	1 14	4 16	8
3½	63 1							
4	3 1	19	J	4 89	16 62	1 17	4 1	3 84
4½	83 0							
5	8? 5	19 4	90 8	4 97	16 61	1 19	4 1	3 79
Recovery Bar								
0 min	4 7							
3	44 5	9 8	6 0	4 04	18 09	1 56	? 55	1 58
G somete								
3 mins	4 8							
5	16 5							
7	24 7							
9	3? 5							
11	8 8							
13	45 8							
15	51 6	46 8	18 3	3 53	1 9	1 06	0	0 60
					B al O c n umpt on		=	0 30

Net O debt in 15 m nutes
3 × (1 58 − 0 0) + 1? (0 60 − 0 30 = 7 48 l ters

Precautions

1 The whole method dep nds on good sampl ng so *be very careful* Rinse out at least 3 times before drawing the final sample
   Accurate timing is almost as important as accurate sampling
3 It will be noted that the two values for the walk should and do che k quite clos ly and sim lar y for the last three minutes of the run Generally a man must run at le st two minutes at this grade of work before the maximum is obtained
4 We have found that the oxyg n d bt *for 15 m nutes is only roughly proportional* to the total oxygen debt For precise measur ment the period should be 60 minutes or until the O consumption approaches the basal level

5 Gasometer readings are taken every half minute during run
6 Expired air samples are taken over an entire minute for the second, third fourth and fifth minutes (the first minute is not of sufficient value) or for as long as the subject runs Samples of less than 20 seconds are of doubtful value

Procedure—Recovery After Exhausting Exercise

1 When subject stops running by jumping to a straddling position (keeping the mouthpiece in and the nose clip on), the following must be done precisely at the time he stops running
 a Valve at treadmill superstructure is turned to collect expired air in Douglas bags for three minutes
 b Cardiotachometer tape is marked.
 c Time is noted
 d Treadmill is stopped Subject sits on tall stool in place on treadmill still breathing through mouthpiece
2 As soon as possible the following additional steps should be taken
 a Final gasometer reading is taken
 b Temperature of expired air in gasometer is noted.
 c Gasometer is emptied Connection removed from mixing chamber and placed on direct connection to gasometer Valve turned for collection and reading noted
3 Blood pressure is taken as soon as possible, preferably during the first minute after subject stops running
4 At the end of three minutes valve at treadmill superstructure is turned to gasometer
5 Gasometer readings are taken every minute during the remainder of the 15 minutes
6 Finger blood samples for blood sugar and lactate are taken at 5 10 and 15 minutes during recovery
7 Cardiotachometer is left running at least for the first five minutes of recovery and may be turned off then and used only to take heart rate during 10th and 15th minutes of recovery
8 At 15 minutes gasometer valve is closed temperature is recorded and final reading is noted
9 Two expired air samples are taken from gasometer (3 15 minutes of recovery)
10 After gasometer is emptied valves are turned connecting Douglas bag to gasometer and initial reading is noted Two samples of contents of bag are drawn from small side tube on bag as gas is forced to large gasometer for measurement of volume Bag is finally rolled to expel all air connection clamped and gasometer volume and temperature are noted (Three minutes of recovery)
11 Barometric pressure is recorded

(The work experiment as carried out above required at least three and preferably four men to guarantee smooth operation.)

Calculations and Data Obtained from Work Experiments

1 Blood pressure at rest (sitting) and immediately following exhausting run
2 *Continuous heart rate record* counted to obtain following
 a Rest (sitting)
 b Maximum during walk (sixth minute)
 c Maximum during run (usually last minute)
 d First five minutes of recovery May be counted by six second intervals for first minute of recovery and longer intervals up to five minutes
 e At 10 minutes during recovery
 f At 15 minutes during recovery

3 Blood sugar and blood lactate in mg per 100 ml
 a During walk
 b At 5 10 and 15 minutes during recovery
4 Metaboli m during walk run and recovery are calculated as described in previous sections
5 Oxygen debt This measurement is the d fference in oxygen consumption for fifteen minutes following the run and the basal requirement for that period To be complete the period should be 60 minutes

Example of a Protocol of a Typical Experiment

SUBJECT B Grade 8 6%	DATE 8/3/49	BAR PRESSURE 761 mm Hg GASOMETER TEMP 34 C			TISS IT FACTOR × cm STP FACTOR 0 896 COMBINED FACTOR 4 69			
WALK 3 5 m p h	SPIROMETER READINGS cm	DIFF cm	PULMONARY VENTIL L/MIN	CO₂ %	O₂	R Q	TRUE O₂ %	O₂ USED L/MIN
4 mins	4 6							
5	13 1	8 5	39	4 50	16 6	0 97	4 70	1 8
6	1 0	9	36 9	4 (1	15 87	0 91	5 17	1 91
Run 10 m p h.								
0 mins	4 5							
1	16 9							
1½	5 6							
2	34 0	1 1	80 0	4 0	16 48	1 06	4 40	3 5
½	44 0							
3	53 4	19 4	90 8	4 9	16 65	1 14	4 16	3 78
3½	63 1							
4	73 1	19	9	4 89	16 6	1 17	4 17	3 84
4½	83 0							
5	8 5	19 4	90 8	4 9	16 61	1 19	4 17	3 9
Recovery								
Bag								
0 min	4 7							
3	44 5	39 9	6 0	4 04	18 08	1 50	2 5	1 58
Gasometer								
3 mins	4 9							
5	16 5							
7	24 7							
9	32 5							
11	38 8							
13	45 8							
15	51 6	46 8	18 3	3 3	1 9	1 06	1 30	0 60
					Basal O consumption		=	0 30

Net O debt in 15 minutes
3 × (1 58 – 0 30) + 1 × (0 60 – 0 30) = 4 9 liters

Precautions

1 The whole method depends on good sampling *so be ve y careful* F u e out at least 3 times before drawing the final sample.
2 Accurate t m ng is almost as important as accurate sampl ng
3 It will be noted that the two values for the walk should and do check quite closely and similarly for the last three minutes of the run. Generally a man must run at least two minutes at th s grade of work before the maximum is obtained
4 We have found that the oxygen debt for 15 minutes is only roughly proportional to the total oxygen debt For *precise measurement* the period should be 60 m nutes or until the O consumption approaches the basal level

SECTION VI

PHYSIOLOGICAL MEASUREMENTS (Continued)

E PULMONARY MEASUREMENTS

1 Alveolar Air in Rest and Work

A. IN REST

Reference

Dill D B Hurxthal L. M. Van Caulaert, C Folling A, and Bock, A. V The Carbon Dioxide Equilibrium in Alveolar Air and Arterial Blood. J Biol. Chem 74 303 313 (Aug) 1927

Principle

Alveolar air is that portion of the air in the lungs that is in contact with the alveolar walls According to the concept of Haldane, the last portion of expired air at the end of a forced expiration may be taken as representative of the alveolar air of the whole lung

Apparatus

1 Alveolar air sampler (see line drawing Fig 50) This is a modification of the Haldane Priestley tube with a three way stopcock between the rubber tube and the mouthpiece The smaller the dead space in the valve the better The rubber tube must be at least 3 feet long
2 A number of gas sampling tubes (Barcroft type) The tube completely filled with mercury including the bore above the stopcock is attached to a sampling outlet on the part of the cock carrying the tube
3 A nose clip
4 A clean mouthpiece
5 A Haldane gas analysis apparatus

Reagents

1 Cl an mercury
2 Reagents for the Haldane gas analyzer

Procedure

1 The mouthpiece and the nose clip are adjusted on the subject after he has rested for at least one half hour
2 The cock has been turned so that the subject is breathing directly into the room.
3 Towards the end of a normal expiration the valve is turned into the rubber tube and the signal 'Blow ' is given
4 The subject expires forcibly and completely without inspiring after the signal Blow is given The tap is immediately turned back so that the subj ct may breathe normally This maneuver leaves a sample of alveolar air in the tube
5 A sample is withdrawn from the tube into a gas sampling tube
6 The sample is then analyzed in the Haldane apparatus for carbon dioxide alone

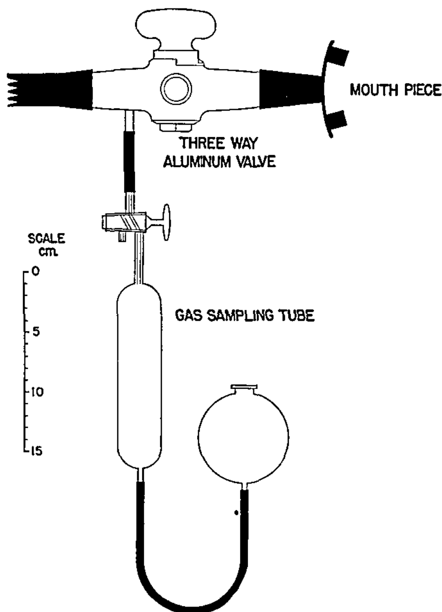


Fig 60 —Arrangement of val es and sampli g tubes fo collecting alveola air by the Haldan P l tley techniqu

PARTIAL PRESSURE OF A GAS FROM BAROMETRIC PRESSURE AND PERCENTAGE OF GAS FOUND

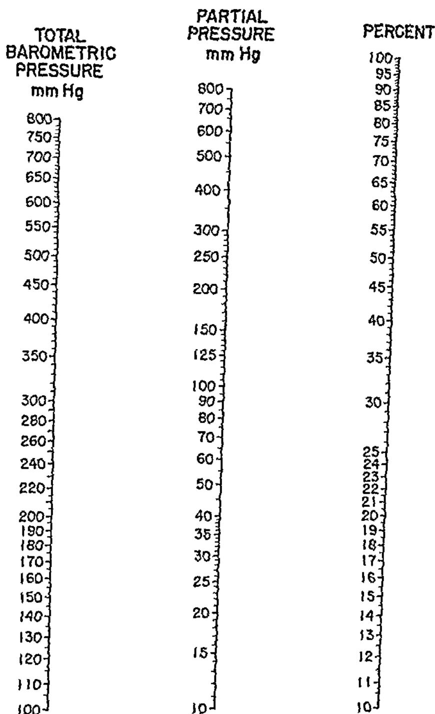


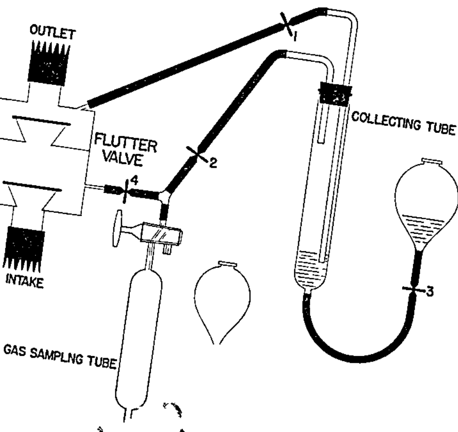
Fig 51 —Nomogram for estimating partial pressure of a gas from barometric pressure and percentage of gas found. Lay ruler to connect the two outside scales at the proper points for total barometric pressure (corrected for tension of aqueous vapor) and percentage of gas found. The partial pressure (mm Hg) of the gas will then be indica ed at the point where the ruler crosse the ce ter scale.

Calculation

Alveolar pCO in mm Hg =

(% CO from Haldane analysis) × $\frac{(B - W)}{100}$

Where B is barometric pressure in mm Hg and W is vapor pressure of water at 37 C i e 47 mm Hg



mu ula ex rci e

2 The line chart Fig 51 gives directly the partial pressure of a gas from the percentage and barometric pressure corrected for vapor tension In the case of CO_2 it is necessary to use some factor such as 10 to enable the right hand scale to be used The reading in the middle scale must be corrected accordingly

Precautions

1 Draw the sample as soon as possible after the expiration
2 Be sure to give the signal Blow only after a normal expiration.
3 Subjects have to be trained in this procedure to give reliable results

B IN WORK

Reference

Henderson Y and Haggard, H W The Circulation and Its Measurement Am J Physiol 73 193 253 (June) 19°5

Principle

During work respiration is not under the same easy voluntary control as it is in rest. Hence the procedure for collecting alveolar air in rest cannot be used in work. A device of Y Henderson and associates collects samples automatically It utilizes the slight negative pressure which is produced during inspiration to suck samples of the last of the preceding expiration into a collecting bottle

Apparatus

1 Y Henderson s apparatus for collecting alveolar air, during exercise (see Fig 5?)
2 Gas sampling tubes (50 ml volume) filled with mercury
3 Other apparatus as for work metabolism

Reagents

1 Clean mercury
2 Reagents for Haldane gas analysis
3 Water acidulated slightly with sulfuric acid for filling the apparatus

Procedure

1 The mouthpiece and nose clip are adjusted on the subject when the metabolic collection period begins
2 All clamps on the alveolar air sampler are closed
3 When it is desired to collect a sample of alveolar air the steps are
   a Attach a gas sampling tube to the rubber outlet
   b Open clamps 1 2 and 4.
   c The depth of the tube in the acidulated water is determined by the depth of the subject s respiration It is usually about 2 5 mm
   d. The air is allowed to flush out the chamber for at least 2 minutes
   e Clamps 1 and 4 are closed.
   f Clamp 3 is opened
   g A sample is obtained by opening the cock of the sampling tube
   h Analysis of carbon dioxide is performed in the Haldane gas analysis apparatus.

Calculation

Same as for alveolar air in rest

Example

Same as for alveolar air in rest

Precautions

Same as for alveolar air in rest and in addition

1 Acidulation of the water in the collecting apparatus is necessary to prevent absorption of CO

2 Functional Residual Air

Reference

Cournand A Baldwin E DeF Darling R C and Richards D W Jr Studies on Intrapulmonary Mixtures of Gases IV The Significances of the Pulmonary Emptying Rate and a Simplified Open Circuit Measurement of Residual Air J Clin Investigation 20 681 689 (Oct) 1941

Principle

Practically all the nitrogen in the lungs is washed out by seven minutes of breathing oxygen The total exp red air is collected measured and analyzed for N_2. The volume of the gas space in the lungs is calculated from these data An appropriate correction is made for nitrogen freed from solution in the blood

Apparatus

1 A 100 liter Tissot gasometer is used with connections as shown in Fig 53 in which the symbols mean the follow ng

M—mouthpiece
Alv—Evacuated gas sampling tubes
V_0 V V V—Three way respiratory valves
F F F F—One way rubber flutter valves.
B_r—Rubber anesthesia bag
T—One hundred liter (Tissot) gasometer
S—Valve and attachment for obtaining gasometer samples

a The main ci cuit is essentially that used fo open circuit measurement of basal metabolism except that the inlet arm comes from a reservoir bag B which is connected to an oxygen tank The valve V is an essential feature but may be improvised in var ous ways It must be near to the mouthpiece M so that there is negligible dead space by it the subjects breathing can be shifted from room air to oxygen in the room a r or side circuit there should be any suitable alveolar sampling device (Pictured in the diagram as evaculated sampl ng tubes inserted through holes in the rubber tubing connection) The recording pen and drum D are useful but unessential parts of the apparatus

b The dead space under the bell of the gasometer T should be measured before us ng the apparatus A satisfactory accuracy within 00 ml may be attained by geometr cal calculation of the space equally easy and more accurate measurement can be made by adding a known volume of oxygen to the dead space volume of oom air through the thermometer socket (not shown on diagram) and analyzing the mixture in the bell

c If V = vol O added DS = dead space under bell a = % N in oxygen supply b = % N in m xture under bell

$$DS = \frac{V\ (b - a)}{(1 - b)}$$

A Van Slyke manometric apparatus should be available for analysis of the high oxygen mixtures A Scholander gas analyzer is equally as good.

Reagents

1 A tank of oxygen
2 Reagents for Van Slyke or Scholander apparatus

Procedure

1 With V turned to the side circuit flush the main circuit and gasometer six consecutive times with oxygen Then turn V to connect main circuit to outside air and maintain an oxygen flow approx mately equal to the subject s ventilation.

2 Have the subject under basal conditions reclining with a one or two pillow head rest Insert the mouthp ece and put on the nose clip

3 Take the initial gasometer reading
4 Turn V to connect to gasometer
5 Observing the subject's respiratory rhythm and being sure that he is breathing easily turn V to the main circuit exactly at the *end of a normal expiration*
6 Have the subject continue breathing oxygen for 7 minutes adjust the oxygen flow as necessary to keep the bag B nearly full
7 During this time prepare the side circuit for alveolar sampling
8 At the beginning of the expiration closest to the seven minute point turn the valve V to the side circuit and instruct the subject to exhale forcibly Take the alveolar sample The subject may then be disconnected

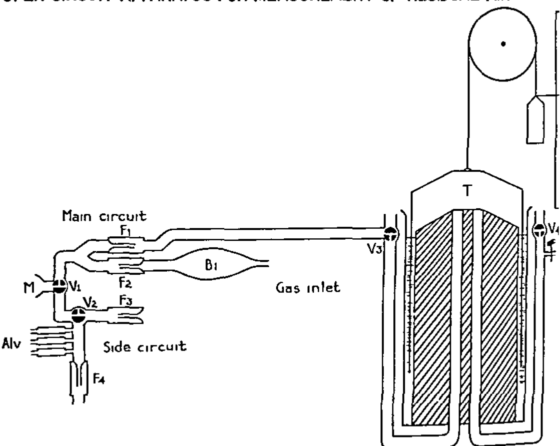


Fig 53 —The apparatus used by Cournand Baldwin Darling and Richards for estimating functional residual air

9 Flush the main circuit with approximately 10 liters of oxygen and close valve V
10 Take second gasometer reading and temperature
11 Take two samples from the gasometer after first flushing the gasometer tubing with the first portion of the contents
12 Repeat steps 1 to 11 for a check determination after ½ hour interval
13 Estimate the nitrogen in the alveolar and spirometer gases using the Van Slyke apparatus or the Scholander gas analyzer

Calculation

Symbols

FRA (dry) = functional residual air in ml (calculated as dry gas)
V = volume of spirometer gas corrected to 37 C (dry)
DS = volume of dead space under gasometer bell corrected to 37 C
a = % N (+ other inert gases) in oxygen supply (usually < 0 5%)
b = % N from gasometer analysis
Alv N = % N in alveolar sample
C = correction for nitrogen from blood (See formula below)
BS = body surface of subject in square meters
BP = barometric pressure mm Hg

Equations

1 $FRA\ (dry) = \frac{(V + DS)\ (b - a) - C}{81 - alv\ N}$

2 Substitute value of C from following

$C\ ml = \frac{(BS \times 96\ 5) + 3}{0\ 80}$

3 For calculation of FRA saturated with water vapor as exists (approximately) within the lungs

$FRA = FRA\ (dry) \times \frac{BP}{BP - 47}$

Example

V was 0 21 l DS 0 2 l a 0 3 % b 8 30% Alv N 3 00% BS 1 75 sq m and BP 760 mm Hg

1 FRA lite s d y =

$\frac{(20\ 1 + 0\ 2)\ (8\ 30 - 0\ 32) - (96\ 5 \times 1\ 75 + 35)/800}{81 - 3}$

= 06

FRA liters wet =

$2\ 06 \times \frac{60}{60 - 47}$

= 2 19

Precautions

This method depends upon

1 Accurate sampling
2 Accurate timing
3 Ext emely accurate analysis of samples of gas high in oxygen

3 Cardiac Output (Grollman s Method)

Reference

Marshall E K Jr and Grollman A A Method fo the Determinat on of the Circulatory Minute Volume in Man Am J Physiol 86 117 137 (Aug) 19 8
Grollman A and Marshall E K Jr The Time Necessary for Rebreathing in a Lung Bag System to Attain Homogeneous Mixture Am J Physiol 86 110 116 (Aug) 19 8

Principle

The subject breathes acetylene which dissolves in plasma but does not combine with hemoglobin lipoids or other constituents of the blood. The quantity absorbed

is measured and from this measurement and the coefficient of solubility of acetylene in plasma the quantity of blood which has passed through the lungs is calculated. The blood flow through the lungs is affected by the rebreathing of acetylene Hence in order to calculate the true cardiac output i e that immediately preceding the breathing Grollman computes arteriovenous difference from the oxygen absorbed during rebreathing and the blood flow during the rebreathing as determined by acetylene absorption The total *oxygen consumption immediately preceding re* breathing divided by the arterio venous oxygen difference gives the cardiac output prior to rebreathing In the analysis of the gas samples three gases are absorbed and a fourth is obtained by difference The analysis is performed in a machine similar in principle to that of the Haldane previously described There are two machines in common use the Grollman Haldane and the Krogh Haldane

Apparatus

1 A Grollman Haldane apparatus This apparatus has an extra absorption tube for the analysis of acetylene
2 A rubber bag connected to a 2 way valve
3 A rack of gas sampling tubes 30 ml volume
4 Apparatus for determining metabolic rate

Reagents

1 A tank of oxygen gas
2 A tank of air
3 A tank of acetylene gas
4 Same reagents as for the Haldane plus an acetylene absorber 20 gm of mer curic cyanide and 8 gm of sodium hydroxide are dissolved and made up to 100 ml of water

Procedure—Estimation of Total Oxygen Consumption

1 Immediately preceding the rebreathing of acetylene estimate the oxygen con sumption of the subject as described under metabolic measurements

Procedure—Rebreathing of Acetylene

1 Depending on the subject s activity fill the bag as follows

	Oxygen	Air	Acetylene
Rest	0	?000 ml	500 700 ml
Activity	500 700 ml	100 500 ml	500 700 ml

2 It is essential that the bag mixture maintain near saturation of the arterial blood throughout the period of rebreathing
3 Prepare a series of evacuated gas sampling tubes
4 The mixture of O N and acetylene is rebreathed from a bag equipped with a 2 way valve and also equipped with two small outlets between bag and valve for taking gas samples
5 The subject with mouthpiece and nose clip in place is instructed to exhale com pletely, at which moment the valve is turned to connect him to the bag contents.
6 He is then instructed to rebreathe the bag contents rapidly and completely but without undue effort The speed should be set so that 5 6 breaths are complete before sample I is taken (See below)
7 It is essential to complete the rebreathing within one circulation of the blood At rest total rebreathing time must be not more than ?0 seconds and in mod erate activity not more than 15 seconds
8 With a resting subject collect Sample I after 10 seconds of rebreathing and Sample II after 18 20 seconds In work take Sample I after 7 seconds and Sample II after 12 seconds

Procedure—Analysis of Samples

1 The procedure for analysis is essentially that of the Haldane machine with a few differences

a After the initial total volume reading the gases are passed into the KOH exactly 8 times no more and a single unchecked reading is obtained. The reason for this is that acetylene is slightly soluble in KOH

b The acetylene is then absorbed. Due to the formation of the white precipitate it is difficult to read the meniscus mark This difficulty is avoided by having a series of marks on the tube and by using these successively beginning with the bottom and ascending as the mark becomes indistinct

c. The oxygen is absorbed as in ordinary gas analysis.

Calculations

1 Volume of lung bag system is reduced during rebreathing since the sum of the O and C_2H_2 absorbed is greater than the CO eliminated N takes no part in the reactions the increase in its percentage is proportional to the decrease in lung bag volume The relative gas volumes at the times of Sample I and Sample II are then

$$V_{II} = V_I \times \frac{\% N_I}{\% N_{II}} \qquad \text{(Equation 1)}$$

2 The amount of C_2H_2 absorbed is proportional to the mean C_2H_2 tension during rebreathing and to the solubility coefficient of C_2H_2 in blood

$$\% C_2H_2 \text{ Av} = \frac{\% C_2H_{2\,I} + \% C_2H_{2\,II}}{2} \qquad \text{(Equation 2)}$$

and ml C_2H_2 absorbed/liter blood during interval between Sample I and Sample

$$\text{II} = \frac{740 \times \% C_2H_2 \text{ Av} \times (B - 47)}{100 \times 760} \qquad \text{(Equation 3)}$$

where 740 is ml C_2H_2 which will be dissolved by blood at 760 mm Hg C_2H_2 pressure at 37 C B is observed barometric pressure and 47 is vapor tension of water at 37 C

3 The blood flow through the lungs is determined by the ratio between total C_2H_2 absorbed during period of observation and the quantity of C_2H_2 absorbed per liter of blood. Liters blood flow through lungs during rebreathing (i.e liters cardiac output during rebreathing) =

$$\frac{(V_I \times \% C_2H_{2\,I} - V_{II} \times \% C_2H_{2\,II}) \times (760 \times 100)}{(740 \times \% C_2H_2 \text{ Av}) \times (B - 47)} \qquad \text{(Equation 4)}$$

4 To obtain cardiac output prior to rebreathing it is now necessary to calculate the arterio venous oxygen difference during rebreathing the quantity of O absorbed by each liter of blood in its passage through the lungs

A − V oxygen difference in ml =

$$\frac{(V_I \times \% O_{2\,I} - V_{II} \times \% O_{2\,II}) \times (740 \times \% C_2H_2 \text{ Av}) \times (B - 47)}{(V_I \times \% C_2H_{2\,I} - V_{II} \times \% C_2H_{2\,II}) \times 760 \times 100} \qquad \text{(Equation 5)}$$

Combining the factors 740 760 and 100

A − V Difference =

$$\frac{(V_I \times \% O_{2\,I} - V_{II} \times \% O_{2\,II}) \times (B - 47) \times 0.00974 \times \% C_2H_2 \text{ Av}}{(V_I \times \% C_2H_{2\,I} - V_{II} \times \% C_2H_{2\,II})}$$

Substituting values from Equation 1

A − V difference =

$$\frac{(V_I \times \% O_{2\,I} - V_I \times \% O_{2\,II} \times \frac{\% N_I}{\% N_{II}}) \times (B - 47) \times 0.00974 \times \% C_2H_2 \text{ Av}}{(V_I \times \% C_2H_{2\,I} - V_I \times \% C_2H_{2\,II} \times \frac{\% N_I}{\% N_{II}})}$$

And V_I cancels out leaving

A − V difference =

$$\frac{(\%O_I - \%O_{II} \times \frac{\%N_I}{\%N_I}) \times \%C_2H_{2A} \times (B - 47) \times 0.0097}{(\%C_2H_I - \%C_2H_{2II} \times \frac{\%N_I}{\%N_{II}})} \quad \text{(Equation 6)}$$

5 Cardiac output, l/min prior to rebreathing =

$$\frac{\text{Total oxygen consumption ml/min}}{\text{A − V oxygen difference ml/l blood}} \quad \text{(Equation 7)}$$

6 Stroke volume ml per heart beat =

$$\frac{\text{Cardiac output l/min}}{\text{Pulse rate beats/min}} \times 1000 \quad \text{(Equation 8)}$$

Precautions

1 This is a *difficult method*. *Much practice* is required on the part of both the observer and the subject to obtain consistent and reproducible results

2 The calculations above derive step by step the final equations which are used in routine calculation Equations 6, 7 and 8

4 Pulmonary and Circulatory Measurements Miscellaneous References

Acetylene

Coulson Smith C and Seyfang A P A Colorimetric Method for the Estimation of Small Quantities of Acetylene in Air, Analyst 67 39 41 (Feb) 1942

Ballistocardiograph

Nickerson J L Symposium on Cardiac Output The Low Frequency Critically Damped Ballistocardiograph Federation Proc 4 (2) 201 206 (June) 1945

Nickerson J L Some Observations on the Ballistocardiographic Pattern With Special Reference to the H and K Waves J Clin. Investigation 28 369 377 (March) 1949

Cardiac Output

McMichael J and Sharpey Schafer E P Cardiac Output in Man by the Direct Fick Method Effects of Posture Venous Pressure Change Atropine and Adrenaline Brit Heart J 6 (1) 33 40 (July) 1944

Ring G C Greisheimer E M Baier H N Oppenheimer M J Sokalchuk A Ellis D and Friday S J Electrokymograph for Estimation of Heart Output Comparison With Direct Fick in Dogs Am J Physiol 161 231 35 (May) 1950

Ring G C, Sokalchuk A, Baier H N, Pudel H Oppenheimer M. J, Friday S J, and Navis G *Electrokymograph for Estimation of Heart Output Comparison With* Stewart in Dogs Am. J Physiol. 161 236 238 (May) 1950

Seely R D and Gregg D E Technic for Measuring Cardiac Output Directly by Cannulation of the Pulmonary Artery Proc Soc Exper Biol & Med. 73 269 270 (Feb) 1950

Warren J V Stead E A Jr and Brannon E S Cardiac Output in Man A Study of Some of the Errors in the Method of Right Heart Catheterization Am. J Physiol. *145 459 464 (Feb) 1946*

Werko L Berseus S, and Lagerlof H A Comparison of the Direct Fick and the Grollman Methods for *Determination of Cardiac Output in Man J Clin Investigation* 28 516 520 (May) 1949

Catheterization

Bing R J Vandam L D, Gregoire F, Handelsman J C Goodale W T and Eckenhoff J E Catheterization of the Coronary Sinus and Middle Cardiac Vein in Man Proc Soc Exper Biol & Med 66 (1) 239 240 (Oct.) 1947

Cournand A Measurement of the Cardiac Output in Man Using the Right Heart Catheterization Description of Technique Discussion of Validity and of Place in the *Study of Circulation Fed Proc 4 207 212 (June) 1945*

Cournand A. and Ranges H A Catheterization of the Right Auricle in Man Proc Soc Exper Biol. & Med. 46 462-466 (March) 1941

Cournand A. Ranges H. A. and Riley R L. Comparison of Results of the Normal Ballistocardiogram and a Direct Fick Method in Measuring the Cardiac Output in Man J Clin. Investigation 21 (3) 287 293 (May) 1942

Cournand A, Riley R L, Breed, E S, Baldwin, E de F and Richards D W Jr Measurement of Cardiac Output in Man Using the Technique of Catheterization of the Right Auricle or Ventricle J Clin Investigation 24 (1) 106-116 (Jan) 1945
Dexter L Haynes F W Burwell C S and Eppinger E C Sagerson R P and Evans J M Studies of Congenital Heart Disease II. The Pressure and O Content in the Right Auricle Right Ventricle and Pulmonary Artery in Control Patients With Observations on the O_2 Saturation and Source of Pulmonary Capillary Blood J Clin Investigation 26 554 560 (May) 1947
Dexter L, Haynes F W Burwell C D Eppinger E C, Seibel R E and Evans J M Studies of Congenital Heart Disease I Technique of Venous Catheterization as a Diagnostic Procedure J Clin. Investigation 26 547 553 (May) 1947

Cerebral Circulation

Kety S S and Schmidt C F The Nitrous Oxide Method for the Quantitative Determination of Cerebral Blood Flow in Man Theory Procedure and Normal Values *J Clin Investigation 27 476 483 (July) 1948*
Kety S S The Quantitative Determination of Cerebral Blood Flow in Man Methods in Medical Research Chicago Vol I 1948 Yearbook Publishers
Myerson A. Halloran R D and Hirsch H L. Technique for Obtaining Blood from the Internal Jugular Vein and Internal Carotid Artery Arch Neurol and Psychiat 17 (6) 8 7 808 (June) 1927

Circulation Time

Wexler J and Whittenberger J L Objective Method for Determining Circulation Time from Pulmonary to Systemic Capillaries by Use of the Oximeter J Clin Investigation 25 (3) 44 450 (May) 1946

Coronary Blood Flow

Eckenhoff J E Hafkenschiel J H Harmel M H Goodale W T Lubin M Bing R J., and Kety S S Measurement of Coronary Blood Flow by the Nitrous Oxide Method Am J Physiol 152 (1) 356 364 (Feb 1948

Electrocardiograph

Graybiel A and White P D Electrocardiography in Practice ed Philadelphia London 1946 W B Saunders Company
Katz L. N Exercises in Electrocardiographic Interpretation ed Philadelphia 1946 Lea and Febiger

Gas Tension in Blood

Roos A., and Black H Direct Determination of Partial and Total Tensions of Respiratory Gases in Blood, Am. J Physiol. 160 163 1 6 (Jan) 1950

Lung Volume

Herrald F J C and McMichael J Determination of Lung Volume A Simple Constant Volume Modification of Christie s Method Proc Roy Soc London 126 (B845) 491 501 (Feb) 1939
McMichael J A Rapid Method of Determining Lung Capacity Clin Sc 4 (3) 167 1 3 (Dec) 1939
Meneely G R and Kaltreider N L Use of Helium for Determination of Pulmonary Capacity Proc Soc Exper Biol & Med 46 (1) 66 69 (Jan) 1941
Meneely G R and Kaltreider N L. The Volume of the Lung Determined by Helium Dilution Description of the Method and Comparison With Other Procedures J Clin Investigation 28 1 9 139 (Jan) 1949
Bateman J B Boothby W M and Helmholz H. F Jr Studies of Lung Volumes and Intrapulmonary Mixing Notes on Open Circuit Methods Including Use of a New Pivoted Type Gasometer for Lung Clearance Studies J Clin Investigation 28 6 9 688 (July) 1949

Pressure Breathing

Barach A L Fenn W O Ferris E L and Schmidt C F The Physiology of Pressure Breathing A Brief Review of Its Present Status J Aviation Med 18 (1) 73 87 (Feb) 1947

Venous Pressure

Lyons R H Kennedy J A and Burwell C S Measurement of Venous Pressure by the Direct Method Am Heart J 16 (6) 6 5 673 (D) 1938

SECTION VI

PHYSIOLOGICAL MEASUREMENTS (Continued)

F PLASMA VOLUME, BLOOD VOLUME, EXTRACELLULAR VOLUME

1 Plasma and Blood Volumes (Dye Method)

Reference

Nitshe G A Jr and Cohen P I Simplified Determination of Blood Volume Am. *J Clin Path* 17 239 243 (*March*) 1947

Principle

The procedure consists of the injection of a known amount of the dye T 1824 (Evans Blue) and the calculation of the plasma volume from the concentration of the dye in the plasma after complete mixing

Apparatus

1 Coleman Jr Spectrophotometer Model 6
2 Cuvettes 10 x 75 mm
3 Specially calibrated syringes sterilized for dye injection
4 10 ml syringes
5 Two 20 gauge needles for venipuncture (sterile)
6 Heparin solution 110 units/mg Dilute 100 mg dry heparin to 14 7 ml isotonic saline and use one drop for the preservation of 10 ml of whole blood
7 0 2 ml syringe pipette accurately calibrated.
8 4 ml hematocrit tubes with cork stoppers to fit
9 Electric centrifuge

Reagents

1 Sterile solution of T 1824 Dye The solution of the dye T 1824 is prepared by dissolving 2 gm in 500 ml distilled water It is passed through a 3 G 3 Jena sintered glass filter and divided between a suitable number of brown bottles and autoclaved Following this rubber diaphragm stoppers are inserted and the bottles stored in the dark Such solutions remain stable and sterile indefinitely when used with reasonable care Each new batch must be calibrated.

Procedure

1 After necessary equipment has been sterilized and thoroughly dried two hematocrit tubes are placed in a rack One drop (0 05 ml) of heparin is placed in each tube Care must be taken that the tubes are chemically clean and that the heparin is not deposited along the rim of the mouth If the tubes are not absolutely clean some hemolysis may result If heparin is deposited at the mouth it may be covered by the cork stopper that is to be used
2 The amount of dye to be injected should be approximately 0 3 mg/kg of body weight The calibrated syringe is filled with the correct quantity of dye
3 With the patient in a reclining position 5 ml of blood is removed from a cubital vein with a dry syringe It is important that there be no stasis of venous blood. Consequently the tourniquet must be removed at least one minute before the blood is drawn into the syringe

4 The measured amount of dye is then injected slowly through the same needle the syringe being filled and emptied at least five times with the patient's blood in order to wash out all traces of dye

5 A notation of the time must be made at the beginning of the injection of the dye While the dye is being injected an assistant should take the freshly drawn blood and fill the first hematocrit tube to the 10 cm mark This should be done quickly but with caution, in order that there be no frothing which may cause hemolysis The cork stopper should be inserted and the tube gently inverted several times until good mixing with the heparin is effected

6 *Approximately nine minutes after beginning the injection of the dye* a clean needle on a clean dry syringe is inserted into a cubital vein The tourniquet is removed and time allowed for venous stasis to correct itself, and when exactly ten minutes have elapsed from the time of injection of dye 5 ml of blood is again withdrawn

7 The needle and syringe are removed and the blood cautiously placed in the second hematocrit tube which is inverted and stoppered in the same manner as before It will be noted that some blood will collect at the junction of the stoppers and the tubes This should be carefully removed with a cotton tipped applicator, the corks likewise wiped off and then replaced

8 Both tubes should then be centrifuged for thirty minutes at 3000 r p m

9 The hematocrit is then read in the conventional manner

10 The supernatant plasma is removed by means of capillary pipettes placed in 10 × 75 mm cuvettes and centrifuged to remove any suspended cells

11 The first cuvette which contains the sample without dye is placed in the spectrophotometer with the wave length set at 6 0 millimicrons The machine is adjusted to 0 density with this sample The second sample which contains dye is then inserted and the density read and recorded.

Calibration Curves

Each batch of dye must be used to make new calibration curves The K value is determined in the presence of plasma at a wave length of 6 0 mμ. The procedure is

1 It is unwise to try to calculate exactly the dye strength in mg/ml because each batch of dye seems to have its own solubility and color characteristics The stock solution contains approximately 4 mg/ml

2 Into 50 ml volumetric flasks pipette exactly 1 and 0 5 ml of stock solution and dilute with distilled water The strengths are approximately 0 08 and 0 04 mg/ml

3 Prepare a batch of 10 15 ml plasma for use in the standardization

4 In separate cuvettes mix 1 8 ml of plasma with exactly 0. ml of diluted dye This gives plasma samples of approximately 0 008 and 0 004 mg/ml

5 Set a control cuvette containing 1 8 ml plasma and 0.2 ml isotonic saline at 0 density at wave length 6 0 mμ

6 Compute K from the formula $K = D/C$ where D is the optical density and C is the dye equivalent in mg/ml C is understood to be an arbitrary figure varying in reality with each batch of dye

Calculation

1 Circulatory plasma volume $\text{ml} = \dfrac{(\text{mg dye injected})}{(\text{mg dye/ml plasma})}$

Where mg dye injected is calculated from the equation (mg dye/ml solution) × (ml injected) and mg dye/ml plasma is calculated from the equation D/K.

2 The total circulating blood volume is calculated from the plasma volume and the hematocrit. One correction has to be made since the hematocrit of peripheral blood does not necessarily equal that of the total blood. Several authors have

BLOOD VOLUME AND CIRCULATING RED CELL MASS FROM OBSERVED PLASMA VOLUME AND HEMATOCRIT

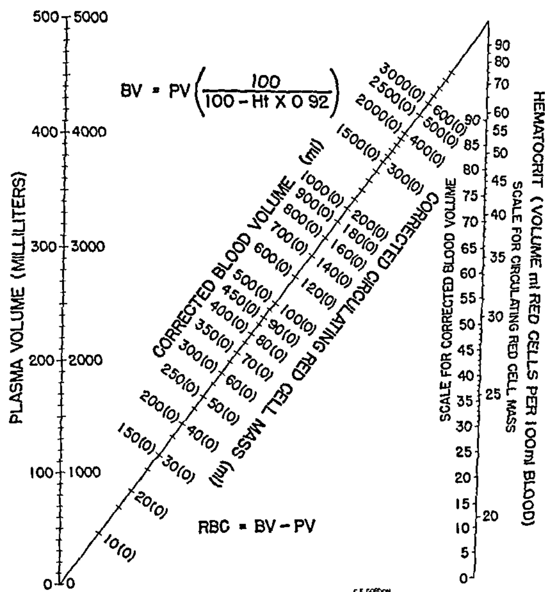


Fig 54 —Line chart for calculating blood volume and circulating red c ll mass from observed plasma volume and hematocrit. Note that *the hematocrit is* multiplied by an arbitrary correction factor of 0 92

urged that for purposes of calculating blood volume the peripheral hematocrit must be corrected by a constant factor 0 92

$$\text{Total circulating blood volume ml} = \frac{(\text{ml plasma volume}) \times (100)}{(100) - (\text{Hematocrit} \times 0\,92)}$$

3 From plasma volume and total circulating blood volume total circulating red cell volume ml = ml total blood volume − ml plasma volume

Example

Into a 0 kg man were injected 1 mg of dye The plasma concentration was 0 0075 mg/ml. Hematocrit was 43 ml %

1 Circulating plasma volume =

$$\frac{21}{0\,0075} = 2800 \text{ ml}$$

2 Corrected circulating total blood volume =

$$\frac{(\ 800) \times (100)}{100 - (43 \times 0\,9\ )} = 463_ \text{ ml}$$

3 Corrected circulating red cell mass =

4632 − 2800 = 1832 ml

4 These calculations are simplified by use of the line chart Fig 54

Precautions

1 The calibration of each new batch of dye is essential for consistent results
2 Hemolysis vitiates the results Although some authors have proposed means of correcting for hemolysis it is our practice to discard any samples in which hemolysis has occurred
3 While extrapolation to zero time by employing a series of successive samples would in all probability give more accurate values it was found that in order to obtain a reliable straight line too many samples were required to make the method practical. Consequently a single ten minute sample was used. The validity of using a single sample has been reported by Gregersen Noble and Miller Hopper Tabor and Winkler and Elkinton Gilder Muller and Phillips have shown that there is complete mixing of the dye with the circulating plasma in this period of time

2 Total Circulating Red Cell, Blood and Plasma Volumes (Carbon Monoxide)

Reference

Root W S Roughton F J W and Gregersen M I. Simultaneous Determinations of Blood Volume by CO and Dye (T 18 4) Under Various Conditions Am J Physiol. 146 739 755 (Aug) 1946

Principle

A measured volume of CO is introduced into the blood by inhalation The increase in CO content of the blood divided into the total amount of CO administered gives the blood volume The individual estimations required are

1 Carbon monoxide content of the blood before and after inhalation of carbon monoxide
Loss of CO if any during and after inhalation
3 Carbon monoxide content and volume of inspired air
4 Residual unabsorbed CO in the expired air

Apparatus

1 Scholander Roughton apparatus for estimating carbon monoxide in blood. Apparatus for generating carbon monoxide

3 Douglas bag apparatus for collecting expired air The method described under metabolic procedures is used with one exception inspiration of outside air is from a six foot length of hose attached to the mouthpiece *inlet valve*

Reagents

1 Those for the Scholander Roughton method of estimating carbon monoxide
2 Carbon monoxide (CO) Place formic acid (HCOOH) in a flask From a dropping funnel add conc sulfuric acid (H SO) Pass the escaping carbon monoxide through a trap filled with pellets of dry KOH and collect in a large bottle by displacing water The stored gas should be 96 to 98% CO

Procedure

1 Allow the subject to lie down for at least 30 minutes
2 Draw a sample of venous blood without stasis from an antecubital vein using heparin for the anticoagulant The blood is collected in a syringe protected from light and stored in the refrigerator
3 Place the nose clip in place and allow the subject to breathe outdoor air through a mouthpiece connected with a 6 foot length of rubber hose one inch in diameter The outlet valve is connected to a 100 liter Douglas bag, which has previously been rinsed well with outside air
4 A 240 ml tonometer containing CO over mercury is connected by means of a short tightly fitted rubber tube with a #20 hypodermic needle, which is thrust into the lumen of the intake rubber hose
5 Immediately after starting to collect the subject s expired air a measured volume of about 240 ml of CO of analyzed purity is introduced into the intake hose over a period of from 3 to 4 minutes
6 Collect expired air for 10 minutes During the last 7 minutes the subject must be inspiring CO free air and the residual CO in his lungs at the end of that time must be negligible compared to that collected in the Douglas bag
7 Allow the subject to rest for 60 minutes more and collect 4 blood samples at timed intervals of 15 minutes from the time the CO was administered.
8 Analyze the expired air and the blood samples for CO using the Scholander Roughton micro technique
9 Mix the gas thoroughly in the Douglas bag and push the volume through a calibrated gasometer The total volume of the expired air is obtained by the difference in gasometer readings
10 Estimate the hematocrit value of the four blood samples and take the average

Calculation

1 Increase in blood CO ml/100 ml =

(ml CO/100 ml at zero time − ml CO/100 ml before inhalation)

where CO at zero time is obtained by extrapolation when the logarithms of the four timed blood specimens are plotted against time

2 Volume of CO inspired dry STP ml =

(ml inject d into tube) × STP fa tor × purity factor

where ml injected is measured STP factor is obtained from the line chart in the section on respiratory metabolism and the purity factor is determined by analysis of the gas administered

3 Total volume of exp red air dry STP is calculated according to the procedure given under the Douglas bag technique from volume wet at room temperature T and ambient barometric pressure

4 CO expired ml, dry STP =

$$\frac{\%\ \text{CO in expired air}}{100} \times \text{volume of exp red air in ml}$$

where % CO is determined by analysis and volume of expired air is calculated as in 3 above

5 CO absorbed ml dry STP = (ml CO inspired – ml CO expired)
where these values are as found in 2 and 4 above
6 Total circulating red cell volume ml =

$$\frac{\text{ml CO absorbed} \times 100}{\text{increase in blood CO ml/100 ml}}$$

7 Total circulating blood volume ml =

$$\frac{\text{red cell volume} \times 100}{\text{average \% hematocrit}}$$

8 Total circulating plasma volume ml =
(total blood volume – total red cell volume)

Example

234 ml of CO was inhaled at 24 5 C and 753 mm Hg STP factor 0 881 The CO was 96% pure total volume of wet expired air was 66 16 l with a CO content of 0 117% The extrapolated zero CO content was 8 10 ml CO/100 ml the content before inhalation was 3 00 ml CO/100 ml The average hematocrit was 42 6 ml/100 ml

1 Increase in blood CO ml/100 ml =
8 10 – 3 00 = 5 10
2 CO absorbed ml =

$$(234 \times 0\ 881 \times 0\ 96) - (66\ 16 \times 0\ 881 \times 1000 \times \frac{0\ 117}{100}) =$$

198 – 68 = 130 ml
3 Total circulating red cell volume ml =

$$\frac{130 \times 100}{5\ 10} = \quad 50\ \text{ml}$$

4 Total circulating blood volume ml =

$$\frac{?5\ 0 \times 100}{4\ 6} = 6000\ \text{ml}$$

5 Total plasma volume ml =
6000 – 2550 = 3450 ml
6 *These calculations may be facilitated by use of the line chart (Fig 54)*

Precautions

1 With small subjects it may be desirable to avoid toxic effects by decreasing the inspired CO to between 150 and 200 ml

3 True Blood Volume

Reference

Same as for dye method and carbon monoxide method for estimating blood volume

Principle

The dye method gives a reliable estimate of circulating plasma volume the carbon monoxide estimates reliably the circulating red cell mass Calculations of blood volume by either method involves empirical corrections of the hematocrit of peripheral blood to make it represent total blood hematocrit By addition of the plasma volume as determined by the dye method and the red cell volume as determined by the carbon monoxide method the true blood volume is calculated

Apparatus

1. Apparatus for plasma volume by the dye method
2. Apparatus for red cell volume by the carbon monoxide method.

Reagents

1 Reagents for the dye method.
2. Reagents for the carbon monoxide method

Procedure

1 Estimate plasma volume by the dye method.
2 Estimate red cell volume by the carbon monoxide method

Calculation

'True blood volume' = ml plasma volume + ml total red cell volume

Example

By the dye method, the plasma volume was 3780 ml By the carbon monoxide method the circulating red cell volume was 2550 ml.

'True blood volume' $= 3780 + 2550 = 6330$

Precautions

Same as for individual methods

4 Extracellular Fluid (Thiocyanate Space)

References

1 Crandall L. A and Anderson M X Estimation of the State of Hydration of the Body by the Amount of Water Available for the Solution of Sodium Thiocyanate Am J Digest Dis & Nutrition 1 126 131 1934
2 Barker M H The Blood Cyanates in the Treatment of Hypertension J A. M A 106 762 767 (March) 1936

Principle

It is alleged that when thiocyanate is injected into the blood stream it becomes distributed uniformly in the interstitial spaces, but not in the cellular mass and that its concentration in plasma after equilibrium is reached is the same as that in the extracellular fluid.

Apparatus

1 *Sterile needles and syringes for intravenous injection*
2 Coleman Jr Spectrophotometer
3 Cuvettes 19 x 150 mm.
4 A quantity of 15 ml round bottom centrifuge tubes with No 1 solid rubber stoppers
5 Syringe pipettes 1 ml and 5 ml.
6 An electric centrifuge

Reagents

1 Trichloracetic acid a 10% solution
2 Ferric nitrate solution. Dissolve 50 gm of ferric nitrate crystals in 600 ml of water *Add 25 ml of concentrated nitric acid and make up to 1000 ml with distilled water*
3 Thiocyanate standard solution (Use potassium or sodium) Dissolve 1 gm KCNS *in 800 ml of distilled water Titrate a 20 ml portion of a silver nitrate solution* (2 9195 gm/1000 ml water) acidified with 5 ml of conc HNO_3 with the KCNS solution using ferric ammonium sulfate as the indicator Calculate the amount of water necessary to add to the KCNS solution to make 20 ml equivalent to 20 ml of silver nitrate solution Add the amount of distilled water and mix thoroughly (1 mg/ml)
4 Thiocyanate for injection. Dissolve 30 gm KCNS in 1000 ml redistilled water *Shake vigorously to dissolve and filter carefully through a sintered glass filter* Store about 11 ml in a 15 ml ampoule and seal. 10 ml = 0 3 gm KCNS Each batch must be analyzed for KCNS
5 Redistilled water

Procedure—Collection of Samples

1 Draw 15 ml of blood for a control sample
2 Now slowly inject intravenously 10 ml of KCNS equivalent to 0 3 gm KCNS and record the time
3 Draw specimens of blood at 1 hour 3 hours and at 24 hours for analysis
4 Just before injection of KCNS have the subject empty his bladder Discard this specimen For the next 24 hours collect urine quantitatively in three separate bottles the first for the period 0 to 1 hour the second for the period 1 to 3 hours and the third for the period 3 to 24 hours
5 Allow each specimen of blood to clot collect serum and analyze the serum and the corresponding urine sample for KCNS

Procedure—Analysis of Serum

1 Pipette 5 ml of trichloracetic acid into a 15 ml round bottom centrifuge tube Add exactly 5 ml of serum shake vigorously and centrifuge
3 Pipette exactly 5 ml of filtrate into a cuvette
4 Add 1 ml ferric nitrate solution
5 For a blank pipette 5 ml of trichloracetic acid into a cuvette and add 1 ml ferric nitrate solution
6 Set blank at 100% T at 550 mμ and read the unknowns.

Procedure—Analysis of Urine

1 Use 5 ml of urine and add 5 ml of trichloracetic acid
2 Mix and pipette 5 ml into a cuvette
3 Add 1 ml ferric nitrate and read at 550 mμ
4 If reading is too high use smaller volume of urine and add more trichloracetic acid.

Calibration Curve

1. Make up a series of standards so that 5 ml contains from 0 10 to 0 50 mg of KCNS
2 Pipette exactly 2 5 ml into a cuvette
3 Add 2 5 ml of trichloracetic acid and 1 ml of ferric nitrate Mix and read at 550 mμ with the blank set at 100% T
4 Plot mg/cuvette vs % T and use this curve in the subsequent calculations.

Calculation—Thiocyanate Concentration

mg KCNS/100 ml serum or urine =

$$\frac{(\text{mg in cuvette}) \times (\text{total dilution}) \times 100}{(\text{mg aliquot}) \times (\text{ml serum or urine used})}$$

Calculation—Extracellular Fluid

kilograms water in extracellular fluid =

$$\frac{\text{ml KCNS injected} - \text{total mg KCNS excreted at time of blood}}{\text{mg KCNS/100 ml serum}}$$

Example

Ten ml of KCNS (0 3 gm) were injected Blood levels at 1 3 and 24 hours were 7 4 and   mg/100 ml. Urine concentrations for the corresponding specimens of urine were 5 15 and 5 mg/100 ml with volumes of 60 00 and 900 ml respectively
kg water in extracellular fluid =
Sample I

$$\frac{300 - \left(5 \times \frac{60}{100}\right)}{7 \times 10} = 4\ 06 \text{ kg}$$

Sample II

$$\frac{300 - \left(25 \times \frac{60}{100}\right) - \left(15 \times \frac{200}{100}\right)}{4 \times 10} = 6.40 \text{ kg}$$

Sample III

$$\frac{300 - \left(25 \times \frac{60}{100}\right) - \left(15 \times \frac{200}{100}\right) - \left(5 \times \frac{900}{100}\right)}{2 \times 10} = 10.5 \text{ kg}$$

Precautions

1 This method has never been proved to measure extracellular fluid.
2 Thiocyanate is a toxic drug Do not inject more than 0 3 gm/day Toxemia may show itself at blood levels over 15 mg/100 ml

5 Plasma Volume Blood Volume and Extracellular Fluid, Miscellaneous References

Blood Volume

Kelly F J Simonsen D H and Elman R Blood Volume Determination in the Human With Red Cells Containing Radioactive Phosphorus (P^{32}) and With Pure Human Albumin J Clin Investigation 27 795 804 (Nov) 1948

Nachman H. M James III G W Moore J W and Evans E I. A Comparative Study of Red Cell Volumes in Human Subjects With Radioactive Phosphorus Tagged Red Red Cells and T 1824 Dye J Clin. Investigation 29 258 264 (Feb) 1950

Nylin G Blood Volume Determinations With Radioactive Phosphorus Brit Heart J *7 81 84 (April) 1945*

Reid Allen and Orr M A Rapid Method for Determining Blood Volumes by the Use of P^{32} Labelled Red Cells J Clin. Investigation 29 313 316 (March) 1950

Cell Volume

Gray S The Use of *Fibrinogen in a Rapid Method of Determining Cell Volume Am* J Med. Sc 207 29 39 (Jan) 1944

Extracellular Fluid

Schwartz I L Measurement of Extracellular Fluid by Means of Constant Infusion Technique Without Collection of Urine Am J Physiol *160 5 6 531 (March) 1950*

Sodium Test

Green D M and Farah A Influence of Sodium Load on Sodium Excretion Am J Physiol 158 444 456 (Sept) 1949

T 1824

Miller A T Jr Excretion of the Blue Dye T 1824 in the Bile Am J Physiol 151 229 233 (Nov) 194

SECTION VII

STUDIES IN THE FIELD

A. GENERAL CONSIDERATIONS

The conduct of scientific surveys is far easier now than it was during most of World War II. When one of the present authors wrote a paper in 1945 summarizing the experience of his colleagues during the war (Johnson, R. E.: A Field Nutritional Laboratory. War Medicine 7: 222-226, April 1945), the scientist had to struggle against difficulties of transportation, lack of permanent construction in many areas to house a field laboratory, the necessity to be self-supporting in almost all respects, and the carrying out of chemical analyses under primitive conditions.

In addition, there was a justifiable suspicion in the minds of many military men in the field that research scientists were a hindrance, not a help, and their cooperation was often given only grudgingly. By comparison, the situation now is almost easy. Everywhere in the world are permanent installations which can be reached in a few days by air. Samples for analysis can be frozen and delivered to a well-equipped base laboratory. Supplies can be obtained by air from the United States very quickly. Finally, most military men realize that the scientists are a help, not a hindrance, and welcome their presence—provided they do not get in the way too much. The construction of well-equipped base laboratories in important areas has been an important step for the biological scientist. Examples are the Canadian laboratory at Fort Churchill, the U. S. Air Force Laboratory at Ladd Air Force Base, and the laboratory supported at Point Barrow by the U. S. Navy.

In spite of the great improvement in conditions, certain basic difficulties remain, of which separation from one's family and friends is one of the most important. The collection, storage, handling and analysis of specimens is still not as easy in the field as it is in university laboratories. Recruitment of able workers presents another difficulty, because in many universities field studies are not regarded as legitimate academic pursuits. It is still the opinion of the present authors that some phenomena can be studied realistically only in the field, whatever the difficulties that must be overcome.

B. LABORATORY AND CLINICAL METHODS

There is no handbook in existence covering field methods for the medical sciences. Each worker must develop his own methods. The present writers feel that field methods should be suitable for use in extremes of temperature

and barometric pressure, *should be as simple as is* consistent with reliability should require the use only of rugged apparatus, and should be rapid enough to handle the large number of specimens which field surveys almost always *accumulate* *Listed below are selected references for chemical and clinical* procedures which are adaptable for medical surveys in the field

1 *Chemical and Clinical Laboratory Methods*

a Many of the methods described in the present manual have been *labelled specifically* as field methods Most of the other methods in the present manual have been used in the field with success

b Bessey and Lowry have devised a system of blood and urine analysis requiring only very small samples Their excellent methods require highly trained technicians and rather expensive apparatus but are otherwise extremely useful for survey work because of their reliability and rapidity

Use of the Beckman Spectrophotometer for Ultramicro Methods

Lowry O H and Bessey O A The Adaptation of the Beckman Spectrophotometer to Measurements on Minute Quantities of Biological Materials J Biol. Chem. 163 *633 639 (June) 1946*

Serum Protein

Lowry O H and Hunter T H Determination of Serum Protein Concentration With a Gradient Tube J *Biol Chem 159 465 475 (July) 1945*

Phosphatase

Bessey O A Lowry O H and Brock M J Method for the Determination of *Alkaline Phosphatase With Five Cubic Millimeters of Serum J Biol Chem* 164 3 1 3-9 (July) 1946

Inorganic Phosphate

Lowry O H and Lopez J The Determination of Inorganic Phosphate in the Presence of Labile Phosphate Esters, J Biol Chem 162 421 428 (March) 1946

Vitamin A

Bessey, O A Lowry O H Brock M J and Lopez J A The Determination of Vitamin A and Carotene in Small Quantities of Blood Serum J Biol Chem 166 177 188 (Nov) 1946

Vitamin C

Lowry O H Lopez J A., and Bessey O A. The Determination of Ascorbic Acid in Small Amounts of Blood Serum, J Biol Chem 160 609 615 (Oct) 1945

Bessey O A A Method for the Determination of Small Quantities of Ascorbic Acid and Dehydroascorbic Acid in Turbid and Colored Solutions in the Presence of Other Reducing Substances J Biol. Chem. 126 771 784 (Dec) 1938

Riboflavin

Lowry O H Bessey O A and Hull T Z Method for the Determination of Five tenths to Two Millimicrograms of Riboflavin J Biol Chem 155 71 77 (Sept) 1944.

2 *Clinical Diagnosis*

The Food and Nutrition Board has produced a monograph entitled Nutrition Surveys—Their Techniques and Value Bulletin of the National Research Council No 17 May 1949 The chapter on clinical diagnosis presents a critical discussion of the clinical study of populations

C TREADMILL TEST OF PHYSICAL FITNESS FOR HARD MUSCULAR WORK

Reference

Johnson R E Brouha L and Darling R C A Test of Physical Fitness for Strenuous Exertion Rev Canad de Biol 1 491 93 (June) 194

Principle

This test is a simple rapid means of assessing the physical fitness of normal healthy men for hard muscular work Any such test must be judged by the following considerations

1 Athletes in training should on the average get much better scores than men not in training

For a large group of untrained men the scores should range widely from bad to good.

3 Normal men not in training should be able to improve their scores by following a program of hard physical work

4 In the case of any given man the score should in general agree with the judgment of trained observers with the results of other reliable tests and most important of all with the man s demonstrated physical capacities in actual performance of hard work

As judged by these criteria certain tests now in common use are of little value in assessing fitness for hard muscular work In *our experience* when *hard muscular* work is in question one must expose the subject to hard work in order to test him accurately This is chiefly because the easier the work the smaller and less regular are the differences between the fit and the unfit Satisfactory assessment of a man s fitness for hard and prolonged work such as marching long distances can be made by subjecting him to a standard exhausting exercise and taking into account only two factors the length of time he can sustain it and his *pulse rate in recovery* after the work is over

Apparatus

1 A motor driven treadmill
2 An accurate stopwatch

Procedure

1 The subject warms up by walking uphill for 5 minutes at 3 5 miles per hour at a grade of 8 6%

He sits on a chair for 5 minutes

3 At a signal he begins to run uphill at a speed of 7 miles per hour and a grade of 8 6% If not exhausted he is stopped at 5 minutes The duration of the run is noted to the nearest second

4 Commence measuring the time of recovery from the time when he stops

5 Count the radial pulses from 1 to 1 5 from 2 to 2 5 and from 4 to 4 5 minutes of recovery

Calculation

Arbitrary score of physical fitness =

$$\frac{\text{(seconds the subject ran)} \times (100)}{(\ ) \times \text{(sum of three half minute pulse rates)}}$$

Example

The subject ran 3 minutes 45 seconds. His three half minute pulse rates were 85 0 and 60 respectively

1 Arbitrary score of physical fitness =

$$\frac{5 \times 100}{_ \times _15} = 5$$

2 The line chart Fig 5 facilitates these computations.

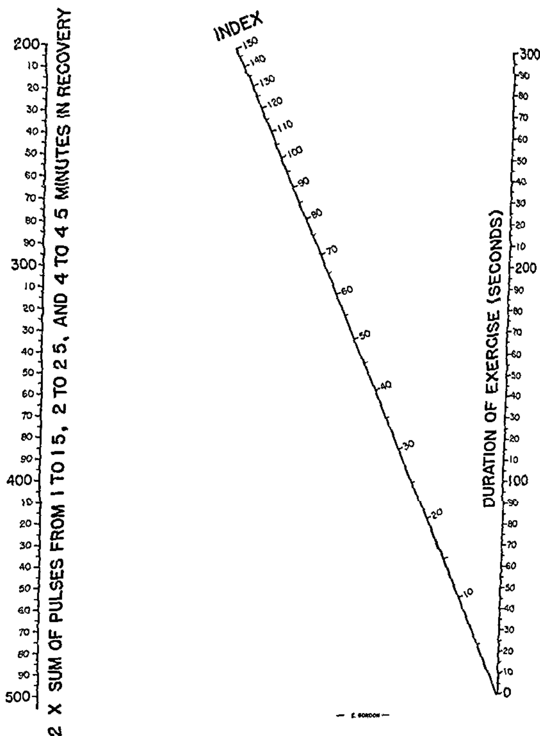


Fig 55.—Calculation of index of physical fitness for hard muscular work in the treadmill, pack and step tests.

Interpretation

From measurements on several hundreds of men of military age of all degrees of fitness the following interpretation of scores is made

A poor score is below 40
An average score is from 41 to 75
A good score is from 76 to 90
A superior score is above 90 Very few men can attain scores above 110

Precautions

1 Any test involving the cooperation of the subject requires the will to work anatomical adequacy to do the work and physiological fitness for the task. A good score generally means that all three factors are good A poor score may mean that any one of the three factors or a combination of them has been weak

D FIELD TESTS OF PHYSICAL FITNESS

1 The Pack Test

Reference

Same as for the treadmill test

Principle

For field purposes the treadmill is not usually available Therefore a modified test is recommended based on the same general prin iple test fitness for hard work by a test that involves hard wo k Some of the accuracy of the treadmill is necessarily sacrificed for the sake of field appl cat on

Apparatus

1 Scales accurate to one pound
2 One watch preferably a stop watch
3 One haversack for each subject
4 A series of bags of metal rock or sand we ghing 10 5 and 1 pound
5 Stepping platform simila to that of the McCurdy Larson test In practice standard gymnasium stools 16 h gh are very good when placed as close as possible to the standard gymnasium stall bars Other platforms can be improvised from boxes or chairs 16 high Ladde s and broomsticks can be used for hand hold One platform is needed for each subject

Procedure for Testing One Subject

1 Weigh the subject stripped or if he is clothed subtract the approximate weight of clothes usually about pounds

Load the pack according to the man s we ght as follows

MAN S WEIGHT STRIPPED	TOTAL PACK WEIGHT
Below 1 0 pounds	40 pounds
1 0 135 pounds	45 pounds
135 150 pounds	50 pounds
1 0 165 pounds	55 pounds
165 180 pounds	60 pounds
180 195 pounds	65 pounds
195 °10 pounds	70 pounds
Over °10 pounds	5 pounds

Note that the pack weight is approximately 1/3 of the subject s body weight.) Be sure that the subject wears a shirt or some other protect on to prevent chafing the shoulders and back. Help him put on the appropriate pack

LINE CHART FOR CALCULATING INDEX OF PHYSICAL FITNES
(HARVARD FATIGUE LABORATORY)

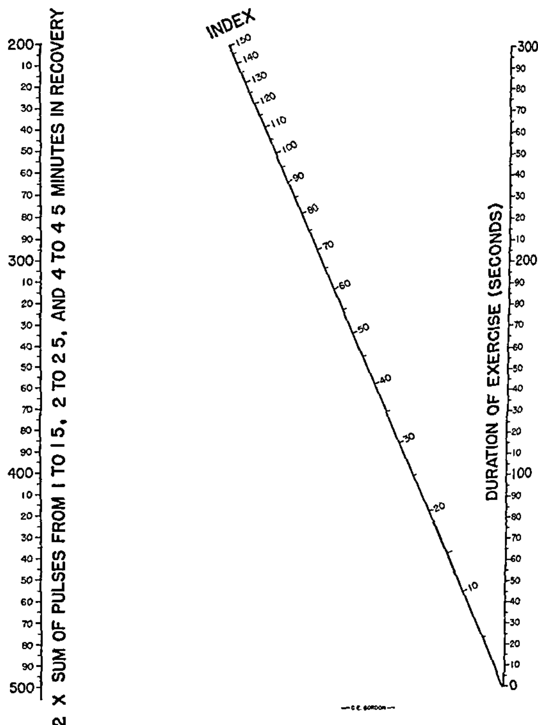


Fig 65.—Calculation of index of physical fitness for hard muscular work in the treadmill, pack and step tests.

4. The times mentioned should be followed as strictly as possible
5. Observers unaccustomed to counting the pulse rate in exercise must practice counting rapid pulses before they can be expected to obtain accurate results.

2 The Step Test

Reference

1 Brouha L Graybiel A and Heath C W Rev Canad de biol 2 86 91 1943
2 Johnson R E Crowley L V Dorsy E A Jr Swan H L Price J I Saffell W B and Jones E O Test of Operational Ration Type E pages 67 70 (March) 1947 Medical Nutrition Laboratory Report #12

Principle

The present test is recommended for use only when it is impossible to use one of the more reliable tests The step test was devised for speed and simplicity of use with large numbers of subjects but a great deal of accuracy has been sacrificed The 20 inch step is so high that it tends to penalize short men and also to involve to some extent the factor of agility as well as general fitness Consequently gross errors may sometimes be made in judging very short or clumsy men However if a lower step is used the work is too easy which so increases the number of errors and impairs the value of the test

Apparatus

1 One watch preferably a stop watch fo each observer
2 A stepping platform 0 inches high (plus or minus not more than ¼ inch) This should be sturdily constructed so as not to give when stepped on It should be at least 14 inches from front to back. When many men are to use the same step simultaneously it should be long enough to allow at least 30 inches for each man. These 30 inch spaces should be marked off Standard gymnasium stools if adjusted to 0 inches in height a e good
3 A device for keeping rhythm The subjects must go up and down every two seconds If a metronome is used it must be a good one that will work without error for 5 minutes We have used satisfactorily a simple pendulum consisting of a weight on a 39 inch string It must be calibrated daily

Procedure

1 As many subjects as there are observe a line up in front of the stepping platform. They should be stripped to the underwear and should wear rubber soled shoes or no shoes at all
One observer calls the rhythm At the signal Start! each subject places one foot on the platform steps up placing both feet on the platform straightens the legs and back and immediately steps down again bringing down first the same foot he placed up first At exactly two second intervals the signal Up! is given and rhythm is maintained by giving the count UP! – 2 – 3 – 4 – UP! – 2 – 3 – 4 etc The subject should lead off with the same foot each time and not try to alternate feet However one or two changes of lead off during the test are immaterial He must not touch anything with his hands but may move his arms freely
3 Begin counting the time when the subject starts exercising and exercise him for five minutes continuously unless he stops before then from exhaustion. If he falls behind stop him after he has been unable to keep up the pace for 20 seconds Note the duration of his effort to the nearest second. All men are stopped at 5 minutes if they can go that long
4. When the subject stops start counting the time and have him sit quietly on a chair.

4. Exercise the subject as follows Have him grasp with both hands a horizontal bar at the height of his shoulders or lightly lower and stand with both feet on the floor The distance between hands should be about the width of his shoulders At the signal UP'' he places one foot on the stool, steps up placing both feet on the stool straightens his legs and back and immediately steps down again bringing down first the same foot he put up first, and retaining his hold on the horizontal bar Every two seconds the signal UP'' is given and the subject steps up and then down He should lead off'' with the same foot each time and not try to alternate the feet However one or two changes of ''lead off in the course of the test are immaterial
5. Begin counting the time when the subject starts and exercise him for five minutes continuously unless he stops from exhaustion before then If he falls behind, stop him after he has been unable to keep the pace for twenty seconds Note the duration of the exercise to the nearest second.
6. When he stops start counting the time then take off the pack and have him sit on a stool or chair
7. Count and record the pulse during each of the following intervals 1 to 1 5 2 to 2 5 and 4 to 4 5 minutes after he stops working The counts must be limited precisely to these intervals The whole test is completed in ten minutes No other observations are necessary

Procedure for Testing a Large Group

1. Procure as many stop watches as there are observers
2. Procure 14 packs and load them as follows

   1 pack weighing 40 pounds for subjects weighing below 120 pounds
   2 packs weighing 45 pounds for subjects weighing 120 135 pounds
   3 packs weighing 50 pounds for subjects weighing 135 150 pounds
   3 packs weighing 55 pounds for subjects weighing 150 165 pounds
   2 packs weighing 60 pounds for subjects weighing 165 180 pounds
   1 pack weighing 65 pounds for subjects weighing 180 195 pounds
   1 pack weighing 70 pounds for subjects weighing 195 210 pounds
   1 pack weighing 75 pounds for subjects weighing above 210 pounds

3. Assign two men the task of weighing the subjects giving them packs and acting as secretaries Start as many subjects exercising all at the same time as there are observers to count the pulse One observer stands behind each subject making sure that he has the proper pack seeing that he keeps the rhythm timing the duration of his effort taking off his pack having him sit down counting his pulse and telling them to the secretaries One of the observers gives the signal Start! and then calls UP! every two seconds With six observers it is possible to test about thirty six subjects every hour

Calculation, Example Interpretation

Same as for treadmill test

Precautions

1. *The height of the platforms should be 16 with an outside tolerance of 0 5*
2. The handhold should be a horizontal bar at shoulder height If it is too high the subject will find the work too easy If it is too low he will find it awkward. Vertical bars can be used but the subject must keep his hands at the level of his shoulders throughout the exercise
3. The rhythm should be maintained as closely as possible It is easy to distinguish between those who are unable to keep the pace because of poor sense of rhythm and those who fall behind because of exhaustion. Only the latter should be stopped. Those with a poor sense of rhythm can be kept to the pace by a tap on the elbow every two seconds

E SURVEYS OF FOOD CONSUMPTION

Reference

1 Berryman G H and Chatfield C Short Method of Calculating Nutritive Value of Diets J Nutrition 25 23 27 (Jan.) 1943
2 Berryman G H and Howe P E Short Method of Calculating Nutritive Value of Diets J Nutrition 27 31 40 (Mar) 1944

Principle

In the field it is often necessary to estimate the average food and nutrient consumption of a group For this purpose a short survey is done over a period of ten days The total food consumption (by groups) is calculated from inventory data corrected for plate and kitchen waste The total nutrient consumption is calculated by adding together the nutrients contributed by each food group

Apparatus and Supplies

1 Scales folding 300 lb capacity
2 Hand tally counter
3 Clip board
4 Ruler
5 At least ten 5 gallon cans or equivalent containers for segregation and weighing of waste both kitchen and plate
6 Slide rule
7 Adding or calculating machine
8 Essential books
   a Handbook of Chemistry and Physics
   b National Research Council Tables of Food Composition
   c Table for converting weights to pounds per man per day
   d. Bowes and Church or other standard book on food composition
   e Master Menu
   f TM 10 412 (Army Recipes)
9 Pads of paper 8 x 10½
10 Small notebooks one for each observer
11 Pencils assorted
1 Paper graph one pad
13 Clips paper
14 3 x 4½ bibliography cards
15 Proper forms
16 Rubber bands
17 One dozen manila folders 8 x 10½

Reagents

None unless it is proposed to collect specimens of food If so see sections on collection of specimens and the individual methods contemplated

Procedure

1 It is recommended that the survey be planned to cover a complete 10 day period Shorter periods may give misleading and erroneous results.
2 Inventory and Issue A complete inventory is made of food stores in the mess on the night before each survey and on the night after the last meal of each survey All items issued during the survey are weighed and recorded.
3 *Head Count* At each meal one observer is stationed near the place where trays are returned. Each man is tallied on a hand counter as he hands in his tray Meals at odd hours especially at night are recorded by night cook and baker
4 Kitchen and Plate Waste Food groups are segregated and weighed in the following categories meats fish and poultry eggs milk products butter other

5 Beginning exactly one minute after he stops count the number of heart beats for exactly 30 seconds The base of the neck is the easiest place to find the pulse after exercise

6 Record the duration of effort and the number of heart beats in the 30 second period beginning one minute after he stops No other observations are necessary

Calculation

A simplified table is used for scoring (Table 2.) From the duration of effort (left hand vertical column) and the heart beats (top horizontal column) read directly the arbitrary score

Example

A subject lasted 3 minutes and 35 seconds His pulse from 1 to 1½ minutes in recovery was 79 beats in the 30 seconds From the table his score was 53

Interpretation

This test is easier to perform than either the 'treadmill or pack'' tests.
For normal healthy young men
Poor is below 50,
Average is 50 through 80,
Good is above 80

Precautions

1 The platform must be 20 inches high
2 The rhythm should be maintained as closely as possible It is easy to distinguish between those unable to keep pace from exhaustion and those who merely have a poor sense of rhythm Only exhausted men should be stopped before five minutes Those with a poor sense of rhythm can be kept to the pace by a tap on the elbow every 2 seconds
3 Times must be followed strictly
4 The subjects must straighten legs and back at each step
5 Observers unaccustomed to counting rapid pulses must practice before they can be expected to obtain accurate results

TABLE 2?

SCORING THE 'STEP TEST OF PHYSICAL FITNESS

Instructions for using the table (1) Find the appropriate line for duration of effort (?) then find the appropriate column of the pulse count (3) read off the score where the line and the column intersect

DURATION OF EFFORT MINS	TOTAL HEART BEATS 1 MIN TO 1½ MIN IN RECOVERY											
	40 TO 44	45 TO 49	50 TO 54	55 TO 59	60 TO 64	65 TO 69	70 TO 74	75 TO 79	80 TO 84	85 TO 89	90 TO 94	95 TO 99
	SCORE (ARBITRARY UNITS)											
0 to ½	6	6	5	5	4	4	4	4	3	3	3	3
½ to 1	19	17	16	14	13	12	11	11	10	9	9	8
1 to 1½	32	29	26	24	22	20	19	18	17	16	15	14
1½ to 2	45	41	38	34	31	29	27	25	23	22	21	20
2 to 2½	58	52	47	43	40	36	34	32	30	28	27	[illegible]5
2½ to 3	71	64	58	53	48	45	42	39	37	34	33	31
3 to 3½	84	75	68	6[illegible]	57	53	49	46	43	41	39	37
3½ to 4	97	87	79	72	66	61	57	53	50	47	45	4[illegible]
4 to 4½	110	98	89	8[illegible]	75	70	65	61	57	54	51	48
4½ to 5	123	110	100	91	84	77	72	68	63	60	57	54
5	129	116	105	96	88	8[illegible]	76	71	67	63	60	56

E SURVEYS OF FOOD CONSUMPTION

Reference

1 Berryman G H and Chatfield C Short Method of Calculating Nutritive Value of Diets J Nutrition 25 3-7 (Jan) 1943
2 Berryman G H and Howe P E Short Method of Calculating Nutritive Value of Diets J Nutrition 27 231-240 (Mar) 1944.

Principle

In the field it is often necessary to estimate the average food and nutrient consumption of a group For this purpose a short survey is done over a period of ten days The total food consumption (by groups) is calculated from inventory data corrected for plate and kitchen waste The total nutrient consumption is calculated by adding together the nutrients contributed by each food group

Apparatus and Supplies

1 Scales folding 300 lb capacity
2 Hand tally counter
3 Clip board.
4 Ruler
5 At least ten 5 gallon cans or equivalent containers for segregation and weighing of waste both kitchen and plate
6 Slide rule
7 Adding or calculating machine
8 Essential books
   a Handbook of Chemistry and Physics
   b National Research Council Tables of Food Composition
   c. Table for converting weights to pounds per man per day
   d. Bowes and Church or other standard book on food composition
   e Master Menu
   f TM 10-412 (Army Recipes)
9 Pads of paper 8 × 10½
10 Small notebooks one for each observer
11 Pencils assorted
12 Paper graph one pad
13 Clips paper
14 3 × 4½ bibliography cards
15 Proper forms
16 Rubber bands
17 One dozen manila folders 8 × 10½

Reagents

None unless it is proposed to collect specimens of food. If so see sections on collection of specimens and the individual methods contemplated

Procedure

1 It is recommended that the survey be planned to cover a complete 10 day period Shorter periods may give misleading and erroneous results.
2 Inventory and Issue A complete inventory is made of food stores in the mess on the night before each survey and on the night after the last meal of each survey All items issued during the survey are weighed and recorded.
3 Head Count At each meal one observer is stationed near the place where trays are returned. Each man is tallied on a hand counter as he hands in his tray Meals at odd hours especially at night are recorded by night cook and baker
4 Kitchen and Plate Waste Food groups are segregated and weighed in the following categories meats fish and poultry eggs milk products butter other

5 Beginning exactly one minute after he stops count the number of heart beats for exactly 30 seconds The base of the neck is the easiest place to find the pulse after exercise

6 Record the duration of effort and the number of heart beats in the 30 second period beginning one minute after he stops No other observations are necessary

Calculation

A simplified table is used for scoring (Table 22) From the duration of effort (left hand vertical column) and the heart beats (top horizontal column) read directly the arbitrary score

Example

A subject lasted 3 minutes and 35 seconds His pulse from 1 to 1½ minutes in recovery was 79 beats in the 30 seconds From the table his score was 53

Interpretation

This test is easier to perform than either the "treadmill" or "pack" tests
For normal, healthy young men
Poor is below 50
Average is 50 through 80
Good is above 80

Precautions

1 The platform must be 20 inches high
2 The rhythm should be maintained as closely as possible It is easy to distinguish between those unable to keep pace from exhaustion and those who merely have a poor sense of rhythm. Only exhausted men should be stopped before five minutes Those with a poor sense of rhythm can be kept to the pace by a tap on the elbow every [illegible] seconds
3 Times must be followed strictly
4 The subjects must straighten legs and back at each step
5 Observers unaccustomed to counting rapid pulses must practice before they can be expected to obtain accurate results

TABLE 22

SCORING THE STEP TEST OF PHYSICAL FITNESS

Instructions for using the table (1) Find the appropriate line for duration of effort ([illegible]) then find the appropriate column of the pulse count (3) read off the score where the line and the column intersect

DURATION OF EFFORT MINS	TOTAL HEART BEATS 1 MIN TO 1½ MIN IN RECOVERY											
	40 TO 44	45 TO 49	50 TO 54	55 TO 59	60 TO 64	65 TO 69	70 TO 74	75 TO 79	80 TO 84	85 TO 89	90 TO 94	95 TO 99
	SCORE (ARBITRARY UNITS)											
0 to ½	6	6	5	5	4	4	4	4	3	3	3	3
½ to 1	19	17	16	14	13	1[illegible]	11	11	10	9	9	8
1 to 1½	32	29	26	24	2[illegible]	20	19	18	17	16	15	14
1½ to	45	41	38	34	31	[illegible]9	27	25	23	2	[illegible]1	[illegible]0
[illegible] to 2½	58	52	47	43	40	36	34	32	30	[illegible]8	[illegible]7	25
2½ to 3	71	64	58	53	48	45	42	39	37	34	33	31
3 to 3½	84	75	68	6[illegible]	57	53	49	46	43	41	39	37
3½ to 4	97	87	79	72	66	61	57	53	50	47	45	4
4 to 4½	110	98	89	82	75	70	65	61	57	54	51	48
4½ to 5	1[illegible]3	110	100	91	84	77	7[illegible]	68	63	60	57	54
5	1[illegible]9	116	10[illegible]	96	88	8	76	71	67	63	60	56

E SURVEYS OF FOOD CONSUMPTION

Reference

1 Berryman G H. and Chatfield C Short Method of Calculating Nutritive Value of Diets J Nutrition 25 23 27 (Jan) 1943
2 Berryman G H and Howe P E Short Method of Calculating Nutritive Value of Diets J Nutrition 27 31 ?40 (Mar) 1944

Principle

In the field it is often necessary to estimate the average food and nutrient consumption of a group For this purpose a short survey is done over a period of ten days The total food consumption (by groups) is calculated from inventory data corrected for plate and kitchen waste The total nutrient consumption is calculated by adding together the nutrients contributed by each food group

Apparatus and Supplies

1 Scales folding 300 lb capacity
? Hand tally counter
3 Clip board.
4 Ruler
5 At least ten 5 gallon cans or equivalent containers for segregation and weighing of waste both kitchen and plate
6 Slide rule
7 Adding or calculating machine
8 Essential books
   a Handbook of Chemistry and Physics
   b National Research Council Tables of Food Composition
   c. Table for converting weights to pounds per man per day
   d. Bowes and Church or other standard book on food composition
   e Master Menu
   f TM 10-412 (Army Recipes)
9 Pads of paper 8 × 10½
10 Small notebooks one for each observer
11 Pencils assorted.
1 Paper graph one pad
13 Clips paper
14. 3 × 4½ bibliography cards
15 Proper forms
16 Rubber bands
17 One dozen manila folders 8 × 10½

Reagents

None unless it is proposed to collect specimens of food If so see sections on collection of specimens and the individual methods contemplated

Procedure

1 It is recommended that the survey be planned to cover a complete 10 day period Shorter periods may give misleading and erroneous results.
2 Inventory and Issue A complete inventory is made of food stores in the mess on the night before each survey and on the night after the last meal of each survey All items issued during the survey are weighed and recorded.
3 Head Count At each meal one observer is stationed near the place where trays are returned. Each man is tallied on a hand counter as he hands in his tray Meals at odd hours especially at night are recorded by night cook and baker
4 Kitchen and Plate Waste Food groups are segregated and weighed in the following categories meats fish and poultry eggs milk products butter other

5 Beginning exactly one minute after he stops count the number of heart beats for exactly 30 seconds The base of the neck is the easiest place to find the pulse after exercise

6 Record the duration of effort and the number of heart beats in the 30 second period beginning one minute after he stops No other observations are necessary

Calculation

A simplified table is used for scoring (Table 22) From the duration of effort (left hand vertical column) and the heart beats (top horizontal column) read directly the arbitrary score

Example

A subject lasted 3 minutes and 35 seconds His pulse from 1 to 1½ minutes in recovery was 79 beats in the 30 seconds From the table his score was 53

Interpretation

This test is easier to perform than either the treadmill'' or ''pack'' tests
For normal, healthy young men
Poor is below 50
Average is 50 through 80
Good is above 80

Precautions

1 The platform must be 20 inches high
2 The rhythm should be maintained as closely as possible It is easy to distinguish between those unable to keep pace from exhaustion and those who merely have a poor sense of rhythm Only exhausted men should be stopped before five minutes Those with a poor sense of rhythm can be kept to the pace by a tap on the elbow every 2 seconds
3 Times must be followed strictly
4 The subjects must straighten legs and back at each step
5 Observers unaccustomed to counting rapid pulses must practice before they can be expected to obtain accurate results

TABLE 2°

SCORING THE STEP TEST OF PHYSICAL FITNESS

Instructions for using the table (1) Find the appropriate line for duration of effort ( ) then find the appropriate column of the pulse count (3) read off the score where the line and the column intersect

DURATION OF EFFORT MINS	TOTAL HEART BEATS 1 MIN TO 1½ MIN IN RECOVERY											
	40 TO 44	45 TO 49	50 TO 54	55 TO 59	60 TO 64	65 TO 69	70 TO 74	75 TO 79	80 TO 84	85 TO 89	90 TO 94	95 TO 99
	SCORE (ARBITRARY UNITS)											
0 to ½	6	6	5	5	4	4	4	4	3	3	3	3
½ to 1	19	17	16	14	13	12	11	11	10	9	9	8
1 to 1½	3°	29	26	24	2°	-0	19	18	17	16	15	14
1½ to 2	45	41	38	34	31	29	27	25	23	[illegible]	21	0
2 to 2½	58	52	47	43	40	36	34	3°	30	28	7	25
2½ to 3	71	64	58	53	48	45	42	39	37	34	33	31
3 to 3½	84	75	68	6°	57	53	49	46	43	41	39	37
3½ to 4	97	87	79	7-	66	61	57	53	50	47	45	4
4 to 4½	110	98	89	8°	75	70	65	61	57	54	51	48
4½ to 5	1 3	110	100	91	84	77	7-	68	63	60	57	54
5	1-9	116	105	96	88	82	76	71	6	63	60	56

E SURVEYS OF FOOD CONSUMPTION

Reference

1 Berryman G H. and Chatfield C Short Method of Calculating Nutritive Value of Diets J Nutrition 25 23-7 (Jan) 1943
2 Berryman G H and Howe P E Short Method of Calculating Nutritive Value of Diets J Nutrition 27 231 240 (Mar) 1944.

Principle

In the field it is often necessary to estimate the average food and nutrient consumption of a group For this purpose a short survey is done over a period of ten days The total food consumption (by groups) is calculated from inventory data corrected for plate and kitchen waste The total nutrient consumption is calculated by adding together the nutrients contributed by each food group

Apparatus and Supplies

1 *Scales folding 300 lb capacity*
2 Hand tally counter
3 Clip board.
4 Ruler
5 At least ten 5 gallon cans or equivalent containers for segregation and weighing of waste both kitchen and plate
6 Slide rule
7 Adding or calculating machine
8 Essential books
   a Handbook of Chemistry and Physics
   b National Research Council Tables of Food Composition
   c Table for converting weights to pounds per man per day
   d. Bowes and Church or other standard book on food composition
   e Master Menu
   f TM 10 412 (Army Recipes)
9 Pads of paper 8 x 10½"
10 Small notebooks one for each observer
11 Pencils assorted.
1 Paper graph one pad
13 Clips paper
14. 3 x 4½ bibliography cards
15 Proper forms
16 Rubber bands
17 One dozen manila folders 8 x 10½

Reagents

None unless it is proposed to collect specimens of food. If so see sections on collection of specimens and the individual methods contemplated

Procedure

1 It is recommended that the survey be planned to cover a complete 10 day period. Shorter periods may give mislead ng and erroneous results.
2 Inventory and Issue *A complete inventory is made of food stores in the mess* on the night before each survey and on the night after the last meal of each survey All items i sued during the survey are weighed and recorded.
3 Head Count At each meal one observer is stationed near the place where trays are returned. Each man is tallied on a hand counter as he hands in his tray Meals at odd hours especially at night are recorded by night cook and baker
4 Kitchen and Plate Waste Food groups are s gregated and we ghed in the following categories meats fish and poultry eggs milk products butter other

5 Beginning exactly one minute after he stops count the number of heart beats for exactly 30 seconds The base of the neck is the easiest place to find the pulse after exercise

6 Record the duration of effort and the number of heart beats in the 30 second period beginning one minute after he stops No other observations are necessary

Calculation

A simplified table is used for scoring (Table 22) From the duration of effort (left hand vertical column) and the heart beats (top horizontal column) read directly the arbitrary score

Example

A subject lasted 3 minutes and 35 seconds His pulse from 1 to 1½ minutes in recovery was 79 beats in the 30 seconds From the table his score was 53

Interpretation

This test is easier to perform than either the ' treadmill or pack ' tests.
For normal healthy young men
Poor is below 50
Average is 50 through 80
Good is above 80

Precautions

1 The platform must be 20 inches high
2 The rhythm should be maintained as closely as possible It is easy to distinguish between those unable to keep pace from exhaustion and those who merely have a poor sense of rhythm Only exhausted men should be stopped before five minutes Those with a poor sense of rhythm can be kept to the pace by a tap on the elbow every 2 seconds
3 Times must be followed strictly
4 The subjects must straighten legs and back at each step
5 Observers unaccustomed to counting rapid pulses must practice before they can be expected to obtain accurate results

TABLE 22

SCORING THE 'STEP TEST OF PHYSICAL FITNESS

Instructions for using the table (1) Find the appropriate line for duration of effort ( ) then find the appropriate column of the pulse count (3) read off the score where the line and the column intersect

DURATION OF EFFORT MINS	TOTAL HEART BEATS 1 MIN TO 1½ MIN IN RECOVERY											
	40 TO 44	45 TO 49	50 TO 54	55 TO 59	60 TO 64	65 TO 69	70 TO 74	75 TO 79	80 TO 84	85 TO 89	90 TO 94	95 TO 99
	SCORE (ARBITRARY UNITS)											
0 to ½	6	6	5	5	4	4	4	4	3	3	3	3
½ to 1	19	17	16	14	13	12	11	11	10	9	9	8
1 to 1½	3	29	26	24	[illegible]	20	19	18	17	16	15	14
1½ to 2	45	41	38	34	31	29	27	[illegible]	23	[illegible]	21	[illegible]
2 to 2½	58	52	47	43	40	36	34	[illegible]	30	28	[illegible]	[illegible]
2½ to 3	71	64	58	53	48	45	[illegible]	39	37	34	33	31
3 to 3½	84	75	68	62	57	53	49	46	43	41	39	37
3½ to 4	97	87	79	[illegible]	66	61	57	53	50	47	45	[illegible]
4 to 4½	110	98	89	82	75	70	65	61	57	54	51	48
4½ to 5	1 3	110	100	91	84	77	72	68	63	60	57	54
5	129	116	[illegible]	96	88	[illegible]	76	71	67	63	60	56

E SURVEYS OF FOOD CONSUMPTION

Reference

1 Berryman G H and Chatfield C Short Method of Calculating Nutritive Value of Diets J Nutrition 25 3 27 (Jan.) 1943
2 Berryman G H and Howe P E Short Method of Calculating Nutritive Value of Diets J Nutrition 27 231 40 (Mar) 1944.

Principle

In the field it is often necessary to estimate the average food and nutrient consumption of a group For this purpose a short survey is done over a period of ten days The total food consumption (by groups) is calculated from inventory data corrected for plate and kitchen waste The total nutrient consumption is calculated by adding together the nutrients contributed by each food group

Apparatus and Supplies

1 Scales folding 300 lb capacity
2 Hand tally counter
3 Clip board
4 Ruler
5 At least ten 5 gallon cans or equivalent containers for segregation and weighing of waste both kitchen and plate
6 Slide rule
7 Adding or calculating machine
8 Essential books
   a *Handbook of Chemistry and Physics*
   b National Research Council Tables of Food Composition
   c Table for converting weights to pounds per man per day
   d. Bowes and Church or other standard book on food composition
   e Master Menu
   f TM 10 412 (Army Recipes)
9 Pads of paper 8 × 10½
10 Small notebooks one for each observer
11 Pencils assorted.
1 Paper graph one pad
13 Clips paper
14. 3 × 4½ bibliography cards
15 Proper forms
16 Rubber bands
17 One dozen manila folders 8 × 10½

Reagents

None unless it is proposed to collect specimens of food. If so see sections on collection of specimens and the individual method contemplated.

Procedure

1 It is recommended that the survey be planned to cover a complete 10 day period. Shorter periods may give misleading and erroneous results.
   Inventory and Issue A complete inventory is made of food stores in the mess on the night before each survey and on the night after the last meal of each survey All items issued during the survey are weighed and recorded.
3 Head Count At each meal one observer is stationed near the place where trays are returned Each man is tallied on a hand counter as he hands in his tray Meals at odd hours especially at night are recorded by night cook and baker
4. Kitchen and Plate Waste Food groups are segregated and weighed in the following categories meats fish and poultry eggs milk products butter other

fats sugars and syrups grain products dry legumes and nuts leafy green and yellow vegetables tomatoes citrus fruits white potatoes, other vegetables other fruits dried fruits condiments, and miscellaneous

For the measurement of plate waste a five gallon can is convenient to use for each food group represented in a particular meal At the end of each meal, each man supervised by an observer segregates his own leftovers according to food groups scraping the food from his tray into the appropriate five gallon can Kitchen waste is also segregated according to food groups and both plate and kitchen waste are weighed. Trap grease is weighed every day and is counted as fat other

Calculation

Calculation although tedious is simple in principle

1 Food intake by food groups =

(First inventory + Food issued) minus

(Last inventory + Kitchen waste + Plate waste)

The breakdown of kitchen and plate waste into groups is made in accordance with the recipes of the various dishes The food groups of canned and other items are determined from recipes or standard tables

2. Nutrient intake by individual nutrients =

(Total food intake, by food groups) × (Group factor for nutrient)

The food group factors may be calculated from tables of food composition such as Bowes A deP and Church, C F Food Values of Portions Commonly Used. A deP Bowes Philadelphia (1946), and U S Department of Agriculture Miscellaneous Publication No 572 Tables of Food Composition in Terms of Eleven Nutrients prepared by the Bureau of Human Nutrition and Home Economics U S Department of Agriculture in cooperation with National Research Council, 1945 Table 23 which follows is a table of nutrient conversion factors used by the Medical Nutrition Laboratory in one of its surveys Cooking losses may be corrected for when the food group factors are computed

3 Food or nutrient intake/man/day =

$$\frac{\text{Total food intake or nutrient intake}}{\text{Average number of men messing} \times \text{total days of survey}}$$

TABLE 23

NUTRIENTS PER POUND OF FOOD GROUPS AS PURCHASED

(Based on food prescribed for the Army, May October 1944 and NRC Tables of Food, Composition Third Revised Edition.)

NUTRIENT	MEATS[1] FISH POULTRY	EGGS FRESH	MILK[2] MILK PRODUCTS	BUTTER	FATS OTHER	SUGARS SYRUPS	CEREALS GRAIN PRODUCTS
Calories/lb	1158	636	4 6	33_7	3860	1730	146
Protein, gm/lb	_9	52	21	3	2	2	49
Fat, gm/lb	102	46	24	368	4_6	0 1	14
Carbohydrate gm/lb	3	3	31	2	4	431	85
Calcium mg/lb	41	218	695	73	_4	"6	217
Phosphorus mg/lb	666	848	558	73	75	13	491
Iron mg/lb	9 0	10 9	0 5	1 0	1 2	2 2	11 9
Vit. A IU/lb[3]	473	4590	1169	17500	263	4	31
Thiamin mg/lb	0 91	0 43	0 18	0 01	0 04	0 01	1 60
Riboflavin, mg/lb[3]	0 84	1 35	1 00	0 05	0 04	0 01	1 "0
Niacin, mg/lb	18 0	0 3	0 6	0 5	0	0 1	13 9
Ascorbic acid mg/lb	6 0	0	6 0	0	0	2 0	0

1. Meat values [illegible] e carcass b[illegible] lean pork cuts Calculate them by the long method.
2 Excludin[illegible] hich is to [illegible] d by the long method The values for milk and milk pro[illegible] a strai[illegible] basis Do not use milk equivalents for calculations.
3 When liv[illegible] 8,000 [illegible] and 12 73 mg riboflavin/lb liver

TABLE 23 (CONT D)

		VEGETABLES				FRUITS		
NUTRIENT	BEANS OTHER DRY LEGUMES NUTS	POTATOES WHITE	TOMATOES	LEAFY GREEN AND YELLOW	OTHER	CITRUS	OTHER FRESH	DRIED
Cal	16 3	3 5	143	1 3	185	15	38	1300
Prot	104	8	5	6	6	3	2	11
Fat	36	0	1	1	1	1	0 8	2
CHO	38	73	8	[illegible]4	38	35	56	308
Ca	530	4	49	1 4	90	9	41	[illegible]
P	1967	13	115	1?8	16	61	57	516
Fe	35 0	2 7	8	3 6	[illegible]	1 1	1 5	18
Vit. A.	12	70	5160	11910	309	3 6	1610	6815
B	0 30	0 40	0 26	0 25	0 15	0 19	0 11	0 13
B_2	1 50	0 15	0 17	0 6	0 18	0 06	0 30	0 69
Niacin	23 9	4 4	3 9	1 7	1 6	0 7	1 4	8 40
C	6 0	57 0	77 0	61 0	40 0	146 0	8 0	8 0

Example

In Table 24 are listed the significant data for a recent survey in Alaska The step by step calculations are not shown but only the final data for food consumption and nutrient intake

Interpretation of Data

If it is desired to compare the results with recommended dietary allowances Table 5 shows the NPC recommended allowances taken from National Research Council Food and Nutrition Board Recommended Dietary Allowances Revised 1948 Reprint and Circular Series No 1 9 (Oct.) 1948 and the Department of Army standards from U S Department of the Army Army Regulation No 40 250 Medical Department Nutrition Washington D C (28 Oct) 1947

Precautions

1 Administrative considerations are important in surveys of food consumption The kitchen and mess personnel must be cooperative and honest enough to give accurate data of inventory and issue

TABLE 24

EXAMPLE OF RESULTS OF A TYPICAL SURVEY OF FOOD CONSUMPTION AND NUTRIENT INTAKE IN A SUBARCTIC[1] CAMP

FOOD GROUP	LBS /MAN/DAY	NUTRIENT	AMOUNT
Meats fish poultry	1 3	Calories	515
Eggs fresh	26	Protein gm	149
Milk & Milk Products	1 13	Fat gm	2 6
Butter	10	Carbohydrate gm	5 1
Fats other	10	Calcium gm	1 ?
Sugars syrups etc	44	Phosphorus gm	3
Cereals	49	Iron mg	6
Legumes	0 01	Vitamin A IU	14 ?00
Vegetables leafy green and yellow	58	Thiamine[2] mg	1 8
Tomatoes	37	Riboflavin[2] mg	3 1
Fruits citrus	[illegible]	Niacin[2] mg	[illegible]3
Potatoes white	86	Ascorbic acid[2] mg	113
Vegetables other	[illegible]		
Fruits other	51		
Fruits dried	03		
Total pounds	6 60		
Waste (lbs kitchen plus plate	89		

[1]Figures represent average/man/day not including intake outside the mess.
[2]Cooking losses calculated according to AR 40-250 page 627

fats sugars and syrups grain products, dry legumes and nuts leafy green and yellow vegetables tomatoes citrus fruits, white potatoes other vegetables *other fruits, dried fruits condiments, and miscellaneous*

For the measurement of plate waste, a five gallon can is convenient to use for each food group represented in a particular meal At the end of each meal each man supervised by an observer segregates his own leftovers according to food groups scraping the food from his tray into the appropriate five gallon can Kitchen waste *is also segregated according to food groups* and both plate and kitchen waste are weighed. Trap grease is weighed every day, and is counted as fat other

Calculation

Calculation although tedious is simple in principle

1 *Food intake, by food groups* =

(First inventory + Food issued) minus

(Last inventory + Kitchen waste + Plate waste)

The breakdown of kitchen and plate waste into groups is made in accordance with the recipes of the various dishes The food groups of canned and other items are determined from recipes or standard tables

2 Nutrient intake by individual nutrients =

(Total food intake by food groups) x (Group factor for nutrient)

The food group factors may be calculated from tables of food composition such as Bowes A deP and Church, C F Food Values of Portions Commonly Used *A. deP Bowes Philadelphia (1946)*, and *U S Department of Agriculture Miscel* laneous Publication No 572 Tables of Food Composition in Terms of Eleven *Nutrients* prepared by the Bureau of Human Nutrition and Home Economics, U S Department of Agriculture in cooperation with National Research Council, 1945 Table 23 which follows is a table of nutrient conversion factors used by *the Medical Nutrition Laboratory in one of its surveys Cooking losses may be* corrected for when the food group factors are computed

3 Food or nutrient intake/man/day =

$$\frac{\text{Total food intake or nutrient intake}}{\text{Average number of men messing} \times \text{total days of survey}}$$

TABLE 23

NUTRIENTS PER POUND OF FOOD GROUPS AS PURCHASED

(Based on food prescribed for the Army May October, 1944 and NRC Tables of Food, Composition Third Revised Edition.)

NUTRIENT	MEATS [1] FISH POULTRY	EGGS FRESH	MILK [2] MILK PRODUCTS	BUTTER	FATS OTHER	SUGARS SYRUPS	CEREALS GRAIN PRODUCTS
Calories/lb	1158	636	4 6	3327	3860	1730	146
Protein gm/lb	59	52	21	[illegible]	2	2	49
Fat, gm/lb	102	46	24	368	4 6	0 1	14
Carbohydrate gm/lb	3	3	31	2	4	431	85
Calcium mg/lb	41	218	695	73	[illegible]4	[illegible]6	[illegible]17
Phosphorus mg/lb	666	848	5[illegible]8	73	75	13	491
Iron mg/lb	9 0	10 9	0 5	1 0	1 2	[illegible]	11 9
Vit A IU/lb [3]	473	4590	1169	17500	263	4	31
Thiamin, mg/lb	0 91	0 43	0 18	0 01	0 04	0 01	1 60
Riboflavin mg/lb [3]	0 84	1 35	1 00	0 05	0 04	0 01	1 0
Niacin mg/lb	18 0	0 3	0 6	0 5	0	0 1	13 9
Ascorbic acid mg/lb	6 0	0	6 0	0	0	2 0	0

1 Meat values exclude carcass beef and all lean pork cuts Calculate them by the long method.

2 *Excluding cheese which is to be calculated by the long method* The values for milk and milk products are on a straight poundage basis Do not use milk *equivalents for calcu* lations.

3 When liver is used add 87000 IU Vitamin A and 1.73 mg riboflavin/lb liver

TABLE 23 (CONT'D)

NUTRIENT	BEANS OTHER DRY LEGUMES NUTS	VEGETABLES				FRUITS		
		POTATOES WHITE	TOMATOES	LEAFY GREEN AND YELLOW	OTHER	CITRUS	OTHER FRESH	DRIED
Cal	18 3	3 5	143	1 8	155	15	38	1310
Prot	104	8	5	6	6	3	[illegible]	11
Fat	56	0	1	1	1	1	0 8	2
CHO	38	73	8	24	38	35	56	308
Ca	530	4	49	174	90	79	41	95
P	1967	213	115	128	167	61	57	516
Fe	35 0	2 7	8	3 6		1 1	1 5	18 2
Vit. A	I	70	5160	11910	308	356	1610	6815
B	30	0 40	0 6	0 25	0 15	0 19	0 11	0 13
B_2	1 50	0 15	0 17	0 6	0 18	0 06	0 30	0 69
Niacin	23 9	4 4	3 9	1 7	1 6	0 7	1 4	8 40
C	6 0	57 0	77 0	61 0	40 0	146 0	8 0	8 0

Example

In Table 24 are listed the significant data for a recent survey in Alaska. The step by step calculations are not shown but only the final data for food consumption and nutrient intak

Interpretation of Data

If it is desired to compare the results with recommended d etary allowanc s, Table 25 shows the NRC recommended allowances taken from National R s arch Council Food and Nutrition Board Recommended Dietary Allowances Revised 1948 Re print and Circular Series No 1 9 (Oct.) 1948 and the Department of Army standards from U S Department of the Army Army Regulation No 40 0 Medical Depart ment Nutrition Washington 25 D C (8 Oct) 1947

Precautions

1 Administrative considerations are important in surveys of food consumption The kitchen and mess per onnel must be cooperative and honest enough to give ac curate data of inventory and issue

TABLE 4

EXAMPLE OF RESULTS OF A TYPICAL SURVEY OF FOOD CONSUMPTION AND NUTRIENT INTAKE IN A SUBARCTIC[1] CAMP

FOOD GROUP	LBS /MAN/DAY	NUTRIENT	AMOUNT
Meats fish poultry	1 8	Calories	15
Eggs fresh	6	Protein gm	149
Milk & Milk Products	1 13	Fat gm	76
Butter	10	Carbohydrate gm	5 1
Fats other	10	Calcium gm	1 2
Sugars syrups etc.	44	Phosphorus gm	2 3
Cereals	49	Iron mg	6
Legumes	0 01	Vitamin A IU	14,00
Vegetables leafy green and yellow	.58	Thiamine[2] mg	1 8
Tomatoes	37	Riboflavin[2] mg	3 1
Fruit citrus	[illegible]	Niacin[2] mg	29
Potatoes white	86	Ascorbic acid[2] mg	113
Vegetables other	[illegible]		
Fruits other	.51		
Fruits dried	03		
Total pounds	6 60		
Waste (lbs kitchen plus plate	89		

Figures represent average/man/day not including intake outside the mess.
[2]Cooking losses calculated according to AR 40-250 page 637

2 The trap grease is important being pure fat
3 The results at best must be *regarded as rough estimates* *This is particularly true of nutrient intake*

Table 25

Recommended Daily Dietary Allowances of the National Research Council, Food and Nutrition Board and Allowances Prescribed by AR 40 250

Nutrient	Food and Nutrition Board: Boys 16 20	Men: Sedentary	Men: Active	Men: Heavy Work	AR 40 250: Sedentary	AR 40 250: Active
Calories[1]	3800	2400	3000	4500	3000	3600
Protein gm	100	70	70	70	100	
Calcium gm	1 4	1 0	1 0	1 0	0 7	
Iron gm	15	12	12	12	-	
Vit A IU	6000	5000	5000	5000	5000	
Thiamine mg	1 7	1 2	1 5	1 8	1 6	
Riboflavin mg	2 5	1 8	1 8	1.8	2.2	
Niacin mg	17	12	15	18	16	
Ascorbic acid mg	100	75	75	75	50	
Vit D IU	400	-	-	-	-	

Calorie allowances must be adjusted up or down to meet specific needs The proper calorie allowance is that which over an extended period of time will maintain body weight or rate of growth at the level most conducive to well being AR 40 250 prescribes 4400 Calories when prevailing temperatures are sub zero for more than 1 week.

F HEAT BALANCE—CLOTHING

Reference

Belding, H S, Darling, R C, Robinson, S Turrell E and Griffin, D R Notes on Methods Used at the Fatigue Laboratory to Evaluate the Thermal Insulation Provided by Cold Weather Clothing, Report No 19 under OSRD Contract No OEM CMR 54 Sept 10 1943

Belding H S Chapter in Clothing Test Methods Edited by Newburgh L. H, and Harris, M 1945

Lee D H K. and Lemons, H Clothing for Global Man, Geographical Review 29 181 213 (No 2) 1949

Importance of Clothing in Field Research

In any study of man in relation to his environment, heat balance is important, and clothing usually has to be taken into account in military studies Clothing affects heat balance through its effects on heat exchange by radiation conduction convection and evaporation convection In addition clothing may affect heat production by its weight or restrictive effects

In relation to radiation effects the clothing may be considered as a barrier between the body and the environment Clothing shuts off about 95% of the radiation from the body which emits in the far infrared Clothing reflects a variable proportion of light incident upon it

The thermal resistance of cloth to conduction is for the most part proportional to the amount of still air held in the substance of the cloth and between its layers For most purposes the nature of the fabric may be neglected and the thermal insulation may be considered as proportional to the total thickness of the clothing assembly Direct conduction to an external object may be increased by compression and wetting of the clothing

2 The trap grease is important being pure fat
3 The results at best must be regarded as rough estimates This is particularly true of nutrient intake

TABLE 25

RECOMMENDED DAILY DIETARY ALLOWANCES OF THE NATIONAL RESEARCH COUNCIL FOOD AND NUTRITION BOARD AND ALLOWANCES PRESCRIBED BY AR 40 250

	FOOD AND NUTRITION BOARD				AR 40 250	
		MEN				
NUTRIENT	BOYS 16 20	SEDENTARY	ACTIVE	HEAVY WORK	SEDENTARY	ACTIVE
Calories[1]	3500	2400	3000	4500	3000	3600
Protein gm	100	70	70	70	100	
Calcium gm	1 4	1 0	1 0	1 0	0 7	
Iron, gm	15	12	12	12	—	
Vit A IU	6000	5000	5000	5000	5000	
Thiamine, mg	1 7	1 2	1 5	1 8	1 6	
Riboflavin mg	2 5	1 8	1 8	1 8	2 2	
Niacin mg	17	12	15	18	16	
Ascorbic acid mg	100	75	75	75	50	
Vit D IU	400	—	—	—	—	

[1]Calorie allowances must be adjusted up or down to meet specific needs The proper calorie allowance is that which over an extended period of time will maintain body weight or rate of growth at the level most conducive to well being AR 40 250 prescribes 4400 Calories when prevailing temperatures are sub zero for more than 1 week.

F HEAT BALANCE—CLOTHING

Reference

Belding H S, Darling, R C, Robinson S, Turrell E, and Griffin, D R Notes on Methods Used at the Fatigue Laboratory to Evaluate the Thermal Insulation Provided by Cold Weather Clothing, Report No 19 under OSRD Contract No OEM CMR 54, Sept 10, 1943

Belding, H S Chapter in *Clothing Test Methods, Edited by Newburgh L H*, and Harris M 1945

Lee, D H K and Lemons H. Clothing for Global Man Geographical Review 29 181 213 (No 2) 1949

Importance of Clothing in Field Research

In any study of man in relation to his environment heat balance is important and clothing usually has to be taken into account in military studies Clothing affects heat balance through its effects on heat exchange by radiation conduction convection and evaporation convection In addition, clothing may affect heat production by its weight or restrictive effects

In relation to radiation effects the clothing may be considered as a barrier between the body and the environment. Clothing shuts off about 95% of the radiation from the body which emits in the far infrared Clothing reflects a variable proportion of light incident upon it

The thermal resistance of cloth to conduction is for the most part proportional to the amount of still air held in the substance of the cloth and between its layers For most purposes the nature of the fabric may be neglected, and the thermal insulation may be considered as proportional to the total thickness of the clothing assembly Direct conduction to an external object may be increased by compression and wetting of the clothing

When the ambient temperature is above skin temperature evaporation is the only avenue of heat loss At all ambient temperatures evaporation must be taken into account When the skin is naked heat of vaporization is drawn from the skin itself and the effectiveness of such evaporation is nearly 100% When clothing is added to the situation there are complications Under hot conditions other avenues of heat are altered the efficiency of convection over the skin is reduced the sweat is evaporated away from the skin part of the heat of vaporization being taken from the ambient air the rate of effective evaporation (i e the evaporation that cools the skin) is reduced and when the whole body has been covered with a film of moisture evaporative cooling has reached its maximum In cold conditions water vapor may recondense in some layer of the clothing assembly returning some of its heat to the clothing system and increasing the thermal conductivity of the fabric by wetting it Under all conditions clothing impedes the diffusion of water vapor outward from the skin textiles differing in this respect one from the other and the movements of the body increase the transfer of heat in the layers of the clothing assembly by a bellows action

The Thermal Insulation of Clothes—The Clo Unit

For histo ical reasons the Clo Un t is important since much of the data on clothing were expressed in terms of Clo units The unit is defined as the thermal insulation necessary to maintain in comfort a sitting resting subject in a normally vent lated room (air movement 0 feet per minute) at a temperature of 70 F and a humidity of less than 50% (Gagge A. I and associates A Practical System of Units for the Description of the Heat Exchange of Man with H s Environment Science 94 4 8-430 June 1941) One Clo unit equals 0 18 C/kilocal/square meter/hour

Experimentally the thermal insulation of a clothing assembly on a man is calculated from the equation

$$\text{Clo value} = \frac{3\,09\ (T \ - T\ )}{H} - I \qquad \text{(Equation 1)}$$

Where T is average skin temperature F

T is ambient temperature F

H , is heat lost through the clothing and

I is the insulation of the air in Clo units at the wind velocity of the test.

T The mean skin temperature is determined by thermocouple measurements of the following areas front of chest lateral aspect of upper arm medial a pect of first phalanx of great toe med al aspect of thigh med al aspect of lower arm, over shoulder blade over gastrocnemius edge of hand at base of first finger and forehead.

I the insulation of air at the wind velocity of the test is obtained from tables compiled by A. C Burton

$$H_{,} = M - S - (F + A) \qquad \text{(Equation 2)}$$

where M is metabolic heat production S is heat stored or lost in the body mass during the experiment E is heat lost by vaporization from lungs and skin and A is heat lost in warming inspired air

M is estimated by standard procedures as described in the sect on on respiratory methods. S is estimated by d rect observation of the change in skin and internal temperatures and calculated according to Burton s equat ons

(1) Mean temperature of body mass =

$$0\,33 \times \text{mean skin temperature} + 0\,67 \times \text{rectal temperature} \qquad \text{(Equation 3)}$$

(2) Stored heat = Δ mean temperature × 0 83 (Equation 4)

0 83 being the specific heat of the body in Cals/kg/ C and Δ mean temperature is in C

E is estimated from the change in the subject s weight (to the nearest gram) corrected for the excess weight of CO expired compared with O consumed *according to the* equation

Cals/square meter/hr vaporized from skin and lungs =

$$\frac{(\text{gm change in weight/hr}) - (0\ 30 \times O_2 l/hr)}{\text{surface area, square meters}} \times 0\ 586 \qquad \text{(Equation 5)}$$

where 0 30 × O_2lhr represents the excess weight of CO_2 expired over O consumed *at R Q 0 88* and *0 586 is the heat of vaporization of water*

A is calculated from pulmonary ventilation as measured by techniques described in previous sections

A (cals/square meter/hour from warming inspired air) =

$$\frac{\text{pulmonary ventilation l/hr} \times 0\ 00031 \times (33\ C - \text{ambient T C})}{\text{surface area square meters}}$$

where 0 00031 is the specific heat of air, kg Cal/cubic meter/ C

Thermal Significance of Clothing

A generalization was made by Burton to express the effect of clothing on heat loss from the surface of the body to a given environment (Burton A C An Analysis of the Physiological Effects of Clothing in Hot Environments Report to Aviation Medical *Research Council of Canada C 2754 SPC 186, Nov 24 1944)*

His equation is

H heat loss in kilocals/square meter/hour =

$$\frac{5 \times W \times (P - P)}{100 \times (r_1 + r)} + \frac{C\ (t - t - RI/C)}{I_1 + I} \qquad \text{(Equation 6)}$$

where

W is % wetted skin

P is vapor pressure of air in mm Hg at skin temperature obtained from tables,

P is vapor pressure of ambient air in mm Hg from standard tables

r_1 and r are respectively the resistance of clothing and air expre ed as cm of still air r_1 is determined experimentally or calculated from the measure thickness of the clothing r from a standard curve which gives the value corrected for the estimated radius of curvature and velocity of ambient air movement

C is a constant 5 55 for one C

t and t are respectively the mean skin temperature and ambient air temperature Measurement of mean skin temperature is described above under ' Clo' unit measurement

I_1 and I are respectively the insulation value of clothing and air, expressed in ' Clo ' units I_1 is determin d experimentally as described in the section on ' Clo units or is calculated at the same time as r_1 I is determined from a standard curve R is absorbed radiant energy in kilocals/square meter/hour

Burton s equation has three limitations it is applicable only to equilibrium conditions it makes no allowances for subclothing convection, and some of the values are difficult to measure or calculate especially the radiant energy factor *It is very useful for predicting* the value of various clothing assemblies or predicting the thermal state of a man wearing a given assembly in a given environment

G METEOROLOGICAL MEASUREMENTS

In all field studies certain meterological data should be collected sys tematically and regularly These include the altitude of the station its lati tude and longitude barometric pressure maximum minimum and ambient *temperatures precipitation hours of sunshine cloud conditions absolute and* relative humidities and wind velocity The charts in the present section are designed to assist in the calculation of such data

Temperature

The first chart (Fig 6) gives direct conversion of Centigrade temperatures into Fahrenheit temperatures and vice versa according to the equation

x F = (9/5 × t C) + 3°
x C = (5/9 × F) − 3

Wind Velocity

The second chart (Fig 57) gives conversion for temperature barometric pressure and wind velocity Wind velocity is converted from meters/second to miles/hour by the equations

x miles/hour ° 369 × (meters per second)
x meters/second 0 4470 × (miles per hour)

Barometric Pressure Altitude and Related Variables

The third chart (Fig 58) gives barometric conversion from millimeters of mercury *to inches of mercury according to the equations*

x millimeters Hg = 25 40 × (inches Hg)
x inches Hg = 0 03937 × (millimeters Hg)

The third chart gives various altitude equivalents From Table 30 Altitude Pressure Tables Based on the United States Standard Atmosphere National Advisory Committee for Aeronautics Report No 538 1935 are g ven the following altitude correlated equiva lents altitude (feet and meters) barometric pressure (inches and millimeters of mer cury) temperature (actual and mean) and oxygen (millimeters partial pressure and equiva lent sea level O c/)

Humidity and Absolute Vapor Tension

The fourth chart (Fig 59) gives the relative humidity from the dry bulb tempera ture and the wet bulb temperatures The fifth chart (Fig 60) for u e in cold areas gives relative humidity from the dry bulb temperature and the difference between the dry bulb and the wet bulb temperatures

It is sometimes necessary to compute absolute vapor tension instead of relative hu midity A line chart (Fig 61) has been constructed for this purpose from the equation

E (absolute vapor tens on in mm Hg) =
$F_w - 0\,0066\ B\ (T_D - T_w)\ [1 + 0\,00115\ (T\ - T_w)]$

where E_w is vapor tens on mm Hg at wet bulb temperature

B is barometric pressure mm Hg
T_D is drv bulb temperature C and
T_w is wet bulb temperature C.

The l ne chart gives vapor tens on for various barometr c pre sure together with the vapor tension from the wet bulb temperature and the difference between the wet bulb and dry bulb temperatures. It mav also be used to compute relative humidity as follows

TEMPERATURE CONVERSION
(CENTIGRADE – FAHRENHEIT)

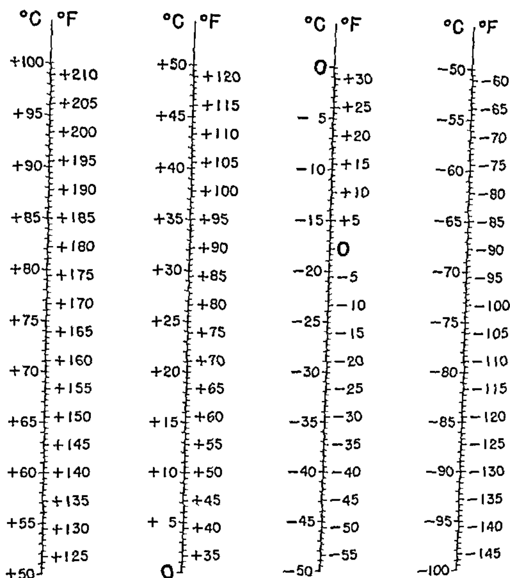
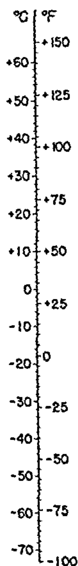
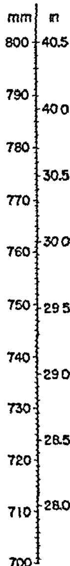


Fig 56 —Line chart for converting Centigrade and Fahrenheit scales

CONVERSIONS, METEOROLOGY





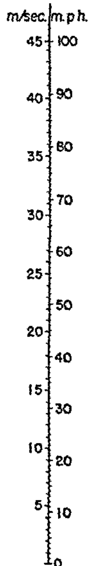


Fig 81—Conversion charts for temperature, barometric pressure and wind velocity

ALTITUDE EQUIVALENTS

ALTITUDE — FEET: 45000, 40000, 35000, 30000, 25000, 20000, 15000, 10000, 5000, 0

ALTITUDE — METERS: 14000, 13000, 12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 0

BAROMETER — INCHES: 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28

BAROMETER — mm Hg: 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700

TEMPERATURE — ACTUAL °C: -55, -50, 45, -40, -35, -30, -25, -20, -15, -10, - 5, 0, + 5, +10, +15

TEMPERATURE — MEAN °C: -30, -25, -20, -15, -10, -5, 0, +5, +10, +15

OXYGEN — EQUIV %: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20

OXYGEN — mm Hg: 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150

G.E. GORDON

Fig 68—Chart constructed for correlated data at different altitudes.

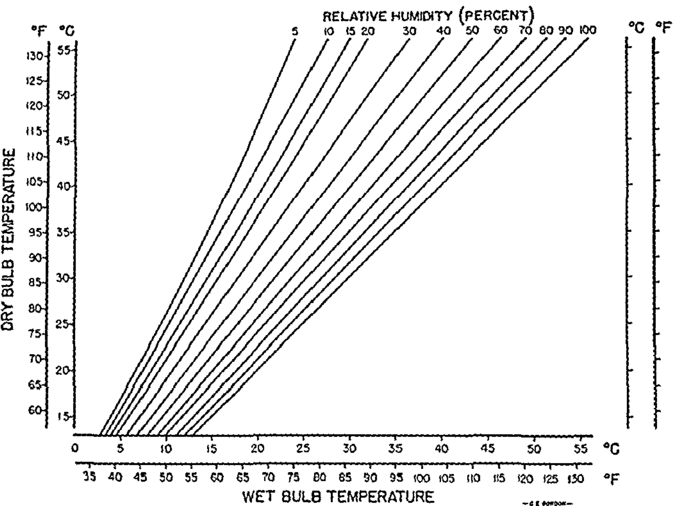


Fig 59.—Cartesian graph for relative humidity from psychrometric data.

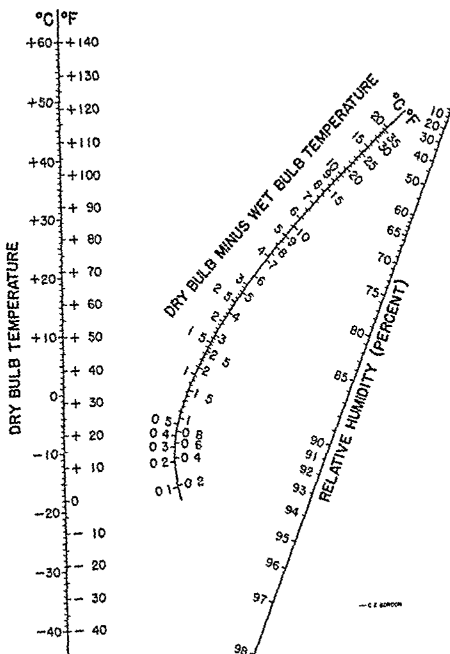


Fig 60—Line chart for relative humidity

ABSOLUTE VAPOR TENSION FROM PSYCHROMETRY

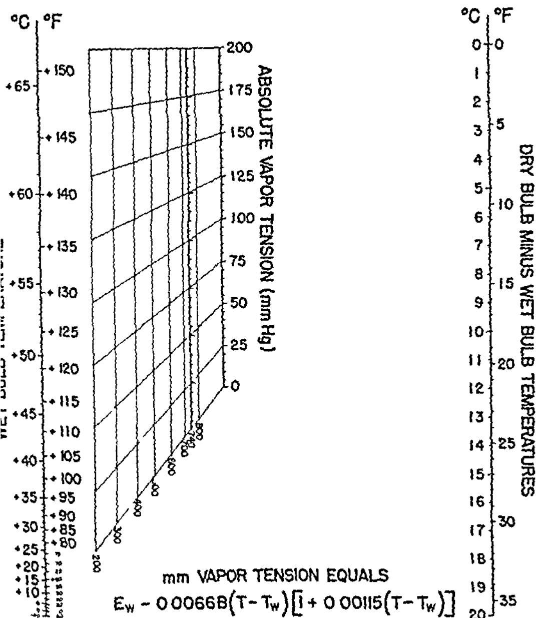


Fig [illegible] — Line chart for computing absolute vapor tension. Measure barometric pressure [illegible] and dry bulb [illegible]. To use the chart (a) stretch ruler between wet bulb line (*L*) and dry bulb minus wet bulb line (*R*) (b) note the point where the ruler intersects the barometric pressure vertical line, (c) turn ruler to run between 0 on the right line and the barometric pressure point (*d*) read absolute vapor tension on center line.

"WINDCHILL"

$$K_0 = \left(\sqrt{WV \times 100} - WV + 10.5\right)\left(33 - T_A\right)$$

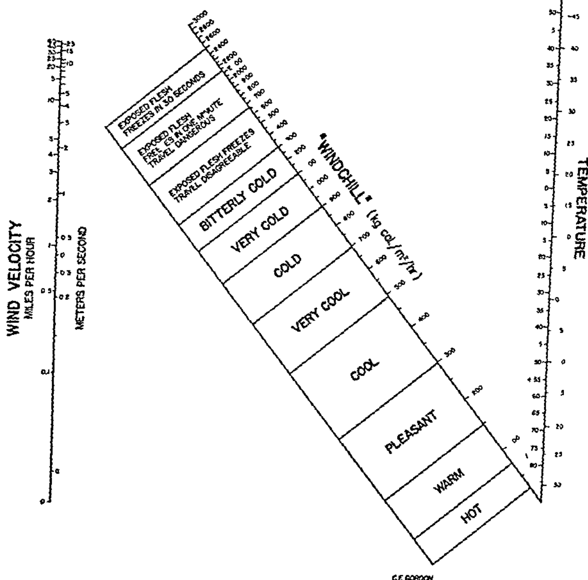


Fig. 6.—Line chart showing "wind hill" and state of comfort.

(a) determine absolute vapor tension, (b) determine vapor tension corresponding to saturation at the dry bulb temperature using the wet bulb scale and a wet bulb-dry bulb difference of 0 (c) calculate relative humidity R.H in % = $\frac{\text{absolute vapor tension}}{\text{vapor tension dry bulb equivalent}} \times 100$

Windchill

The concept of windchill has been very useful in expressing the relative discomfort of cold climates in relation to absolute temperature and wind velocity The quantitative aspects of this concept have been discussed by Siple and Passel (Siple P A. and Passel C F Measurements of Dry Atmospheric Cooling in Subfreezing Temperatures Proc Am Phil Soc 89 177 1945) and Court [Court Arnold Windchill Bull. Am. Meteorol. Soc 29 487-493 (Dec) 1948] The mathematical validity of the equation of Siple and Passel is questionable but nevertheless the concept emphasizes the most important single avenue of body heat loss under windy conditions—convection to the air

The accompanying line chart (Fig 6) enables one to derive windchill from wind velocity and ambient temperature and to estimate at the same time the degree of discomfort of the environment The equation used was that of Siple and Passel

Windchill kilogram Cal/square meter/hour =

$$(\sqrt{WV \times 100} - WV + 10.45) \times (33 - T)$$

where WV is wind velocity meters/second

10 45 is an arbitrary constant

33 C is the average skin temperature and

T is the ambient dry bulb temperature C

H PRESERVATION OF ANIMAL TISSUES FOR HISTOLOGICAL STUDIES

(This section was prepared by K Sutherland and C L Pirani)

References

Lillie R D Histopathologic Technic ed 2 Philadelphia 1948 The Blakiston Company

Glick D Techniques of Histo and Cytochemistry ed. 1 New York 1949 Interscience Publishers Inc

U S War Dept Methods for Laboratory Technicians TM 8 227 War Department Technical Manual 1946

Cowdry E V Laboratory Technique in Biology and Medicine ed. 2 Baltimore Md 1948 Williams and Wilkins Company

Gersh I Application in Pathology of the Method of Fixation by Freezing and Drying of Tissues Bull. Internat Assoc Med Museums 28 1 9 185 1948

During field studies it may be necessary or desirable to study tissues from either animal or human sources The tissues may have been obtained at autopsy or by bioptic or surgical methods If proper precautions in the preservation and fixation of tissue specimens are observed histological histochemical and chemical studies may be carried out and contribute substantially to the understanding of physiological and physiopathological processes Application of such techniques undoubtedly could be very useful in metabolic and nutritional studies that might be conducted in the field

It is beyond the purpose of this manual however to give detailed techniques which can be applied to investigations of this type It is obvious that

in most cases equipment necessary to carry out these methods will not be available in field studies The reader is referred to a number of excellent manuals and textbooks which are listed among the references for detailed histological and histochemical techniques This section will therefore be limited to a brief review of the best available methods to preserve tissues which can be applied to field conditions and which will then permit either storage or shipment of tissue specimens

1 *Preservation of Tissue Specimens for Histological Studies* Probably the best fixative for all around purposes is 10 per cent formalin This fixative is prepared by diluting 1 volume of 40% Formaldehyde (U S P) with 9 volumes of distilled water If distilled water is not available tap water can be used satisfactorily

Blocks of tissue should not be more than 0 4 to 0 5 cm in thickness and 2 to 3 cm in width to insure proper fixation and to prevent distortion The proportion of the formalin solution to volume of tissue to be fixed should be no less than 10 1 In addition it is advisable to change the formalin solution after 24 hours If these simple precautions are used tissues can be kept indefinitely in this fixative

Formalin fixation can be used satisfactorily for most routine histological methods It is not adequate however for many histochemical techniques except those for non water soluble substances such as lipids According to Lillie proper treatment of formalin fixed tissue makes possible the use of additional special staining techniques

In general the use of other fixatives is not advisable under field laboratory conditions unless tissues can be shipped to reach a processing laboratory within 24 hours An important exception is 80% alcohol in which tissues can be kept indefinitely In addition this fixative offers the advantage of freezing at a temperature lower than that of 10% formalin

2 *Preservation of Tissue Specimens for Histochemical and Chemical Studies* Deep freezing of tissue specimens is best suited for this purpose This can be accomplished either by storing the specimens in a deep freezing apparatus or by placing them in a thin walled container which is then surrounded by crushed dry ice Tissues can be kept indefinitely in a deep freeze state without any significant chemical change taking place If shipment of frozen specimens is desired the following precautions are necessary (a) Place specimens in an insulated container such as a vacuum bottle and pack dry ice all around them (b) The volume of dry ice should be at least 25 times greater than that of the specimen This will insure its preservation for a period of no more than 24 hours (c) Make sure that the container is not gas tight and will allow the expanding carbon dioxide to escape

Defrosting of frozen tissue specimens at room temperature is satisfactory for most chemical analysis except when labile substances (for example ascorbic acid and some enzymes) are involved In such case adequate precautions should be used as indicated by the different analytical methods

For histochemical studies neither slow nor quick defrosting can prevent some degree of distortion and loss of histological detail Paraffin embedding of frozen specimens under vacuum in the Altman Gersh apparatus is without doubt the method of choice should such apparatus be available With this technique tissues undergo practically no chemical or histological changes and the blocks obtained can be permanently stored for future slides

3 *Labeling and Packing of Tissue Specimens* Wide mouthed bottles sealed with paraffin should be generally used for tissues in fixing solution (wet tissue) The bottle should be adequately labeled on the outside A second small label of heavy drawing paper written in soft pencil (avoid ink) should be put in the bottle with the specimen Adequate packing into a mailing case marked Fragile First Class Mail Rush Specimen for Diagnosis is satisfactory for shipment Reference is made to the Technical Manual 8 227 of the War Department for additional information on this important phase of the handling of specimens

SECTION VIII

TECHNIQUES FOR METABOLIC WARDS

A GENERAL CONSIDERATIONS

References

Reifenstein E C, Jr, Albright F and Wells S L The Accumulation, Interpretation and Presentation of Data Pertaining to Metabolic Balances Notably Those of Calcium Phosphorus and Nitrogen J Clin Endocrinol 5 367 395 (Nov) 1945

The Physical Arrangement of a Metabolic Ward

Numerous authors have discussed the ideal arrangement of a metabolic ward The paper of Reifenstein Albright and Wells gives a good bibliography on this subject Our own experience has been that the work is greatly facilitated by a well organized physical plant Ideally the patients should have private rooms and in addition a bathroom and common recreation room On the same floor and adjacent to the ward should be

1 A dining room
2 A diet kitchen
3 An office for the staff
4 A record office with a calculating machine
5 A room for preparing and storing specimens
6 A laboratory for routine clinical pathology
7 A separate laboratory for the many special procedures required in metabolic work

All space should be air conditioned because it is becoming increasingly clear that environmental control is extremely desirable for the most consistent results In addition, the comfort of the staff which is usually on a 12 month schedule, is very important for efficient work

Finances of a Metabolic Ward

Few persons not actively engaged in metabolic work realize the large expenses involved For patient care alone in 1949 the cost in Chicago was of the order of $14 per patient per day Add to this the numerous and sometimes expensive laboratory procedures and the cost is such that metabolic work should not be started without an assured adequate budget

Collection Preparation and Storage of Specimens

These topics have been discussed in previous parts of this manual If at all feasible it is better to use analytical data for food consumption rather than calculated data Our experience with stool nitrogen is that for a given patient

within wide limits the daily stool nitrogen is quite constant but that different patients have greatly different characteristic excretions Therefore it is unsound to use calculated values for stool nitrogen

B DERIVED DATA— THEORETICAL BALANCES

1 Calcium, Phosphorus and Nitrogen

The concept of theoretical balances has been extensively used by Albright and colleagues (see reference previously cited) By a theoretical balance is meant the balance of an element such as phosphorus which would be expected from the observed balances of other elements such as nitrogen and calcium The two chief virtues of such derived data are their assistance in detecting gross errors in the data and their emphasis on situations where the measured balances differ from the theoretical

For computation of theoretical calcium phosphorus and nitrogen balances one from the other the following factors are used

Calcium/phosphorus in bone	=	2.23
Nitrogen/phosphorus in muscle	=	14.7 15.5

In computing the balances the following equations are used assuming that muscle is characteristic of total protoplasmic mass

a Theoretical phosphorus from calcium and nitrogen =

$$\frac{\text{gm calcium retained}}{3} + \frac{\text{gm nitrogen retained}}{15}$$

b Theoretical calcium from phosphorus =
(gm phosphorus retained × 2.23)

c Theoretical nitrogen from phosphorus and calcium =

$$(\text{gm phosphorus retained} \times 14.7) - \left(\frac{\text{gm calcium retained}}{2.23} \times 14.7\right)$$

2 Potassium and Sulfur

From potassium sulfur and nitrogen it is possible to calculate theoretical balances The factors used are

mEq potassium	=	2.7 × gm nitrogen
mg potassium	=	10 × gm nitrogen
gm sulfur	=	$\frac{\text{gm nitrogen}}{14.5}$

To calculate the theoretical balances the following equations are used (note that in these cases the results are not additive as they are for Ca P and N)

a Theoretical nitrogen from potassium = $\frac{\text{mEq K retained}}{2.7}$ or $\frac{\text{mg K retained}}{10}$

b Theoretical nitrogen from sulfur = gm S retained × 14.5

c. Theoretical mEq potassium from nitrogen = gm N retained × 2.7

d. Theoretical mg potassium from nitrogen = gm N retained × 105

e Theoretical mEq potassium from sulfur = gm S retained × 14.5 × 2.7

SECTION VIII

TECHNIQUES FOR METABOLIC WARDS

A GENERAL CONSIDERATIONS

References

Reifenstein E C, Jr Albright F and Wells S L The Accumulation, Interpretation and Presentation of Data Pertaining to Metabolic Balances Notably Those of Calcium Phosphorus and Nitrogen J Clin Endocrinol 5 367 395 (Nov) 1945

The Physical Arrangement of a Metabolic Ward

Numerous authors have discussed the ideal arrangement of a metabolic ward The paper of Reifenstein Albright and Wells gives a good bibliography on this subject Our own experience has been that the work is greatly facilitated by a well organized physical plant Ideally the patients should have private rooms and in addition a bathroom and common recreation room On the same floor and adjacent to the ward should be

1 A dining room
2 A diet kitchen
3 An office for the staff
4 A record office with a calculating machine
5 A room for preparing and storing specimens
6 A laboratory for routine clinical pathology
7 A separate laboratory for the many special procedures required in metabolic work

All space should be air conditioned because it is becoming increasingly clear that environmental control is extremely desirable for the most consistent results In addition the comfort of the staff which is usually on a 12 month schedule, is very important for efficient work

Finances of a Metabolic Ward

Few persons not actively engaged in metabolic work realize the large expenses involved For patient care alone in 1949 the cost in Chicago was of the order of $14 per patient per day Add to this the numerous and sometimes expensive laboratory procedures and the cost is such that metabolic work should not be started without an assured adequate budget

Collection, Preparation and Storage of Specimens

These topics have been discussed in previous parts of this manual If at all feasible it is better to use analytical data for food consumption rather than calculated data Our experience with stool nitrogen is *that for a given patient*

within wide limits the daily stool nitrogen is quite constant but that different patients have greatly different characteristic excretions Therefore it is unsound to use calculated values for stool nitrogen

B DERIVED DATA— THEORETICAL BALANCES

1 Calcium, Phosphorus and Nitrogen

The concept of theoretical balances has been extensively used by Albright and colleagues (see reference previously cited) By a *theoretical balance is* meant the balance of an element such as phosphorus which would be expected from the observed balances of other elements, such as nitrogen and calcium The two chief virtues of such derived data are their assistance in detecting gross errors in the data and their emphasis on situations where the measured balances differ from the theoretical

For computation of theoretical calcium phosphorus and nitrogen balances one from the other the following factors are used

Calcium/phosphorus in bone	=	2 3
Nitrogen/phosphorus in muscle	=	14 15 5

In computing the balances the following equations are used assuming that muscle is characteristic of total protoplasmic mass

a Theoretical phosphorus from calcium and nitrogen —

$$\frac{\text{gm calcium retained}}{3} + \frac{\text{gm nitrogen retained}}{15}$$

b Theoretical calcium from phosphorus =
(gm phosphorus retained × 2 23)

Theoretical nitrogen from phosphorus and calcium =

$$(\text{gm phosphorus retained} \times 14\ 7) - \left(\frac{\text{gm calcium retained}}{3} \times 14\ 7\right)$$

2 Potassium and Sulfur

From potassium sulfur and nitrogen it is possible to calculate theoretical balances The factors used are

mEq potassium	=	7 × gm nitrogen
mg potassium	=	105 × gm nitrogen
gm sulfur	=	$\frac{\text{gm nitrogen}}{14}$

To calculate the theoretical balances the following equations are used (note that in these cases the results are not additive as they are for Ca P and N)

a Theoretical *nitrogen from potassium* = $\frac{\text{mEq K retained}}{7}$ or $\frac{\text{mg K retained}}{105}$

b Theoretical nitrogen from sulfur = gm S retained × 14.5
c Theoretical mEq potassium from nitrogen = gm N retained × 2 7
d Theoretical mg potassium from nitrogen = gm N retained × 105
e Theoretical mEq potassium from sulfur = gm S retained × 14.5 × 2 7

f Theoretical mg potassium from sulfur = gm S retained × 14 5 × 105

g Theoretical sulfur from nitrogen = $\frac{\text{gm nitrogen}}{14\ 5}$

h Theoretical sulfur from potassium = $\frac{\text{mEq K retained}}{2\ 7 \times 14\ 5}$ or $\frac{\text{mg K retained}}{105 \times 14\ 5}$

C DERIVED DATA— THEORETICAL WEIGHT

Albright and colleagues have used the concept of theoretical weight" as a means of discussing the causes of shifts of body weight The conditions under which their calculations are valid are

1 The caloric output must be constant
2 The state of hydration and saturation with glycogen must be the same at the beginning and end of the study
3 In other words the patient must be in the same state of equilibrium at the beginning and end of the study The calculations become more accurate the longer the study

In principle one computes the following changes in weight from meta bolic data

1 Change in protoplasmic mass associated with nitrogen, from nitrogen balance
2 Change in weight of extracellular fluid not accounted for with proto plasm from sodium or chloride balance
3 Change in weight of intracellular fluid glycogen or both not accounted for with protoplasm from potassium balance

The difference between the observed change in weight and the sum of the above three separate changes in weight is related to the caloric dis crepancy ' or the weight of fat which would be necessary to account for the change in weight on the basis of energy alone

To carry out the actual calculations Table 26 gives the necessary con version factors

The steps in the calculation are

1 Net change in body weight gm =
Body wt in gm at beginning − Body wt in gm at end
2 Change in weight of protoplasm fat free but not extracellular fluid free related to nitrogen gm =
Nitrogen balance gm × 32
3 Change in weight in grams of extracellular fluid not accounted for with protoplasm =

$$\frac{(\text{Sodium balance mEq})}{(151 \times 1000)} - (\text{Nitrogen balance, gm} \times 5)$$

$$\frac{(\text{Chloride balance mEq})}{(104 \times 1000)} - (\text{Nitrogen balance gm} \times 5)$$

4 Change in weight in grams of intracellular fluid and/or glycogen not accounted for with protoplasm =

$$\frac{(\text{Potassium balance mEq})}{(143 \times 1000)} \quad (\text{Nitrogen balance gm} \times 19)$$

5 Theoretical weight change assuming caloric balance grams =
Sum of changes in weight due to protoplasm extracellular fluid and intracellular fluid

6 Caloric discrepancy i.e grams fat required to account for difference between observed and theoretical weight change =
(Observed net weight change) – (theoretical weight change)

TABLE 6

FACTORS USED IN DERIVING CERTAIN COMPONENTS OF MUSCLE PROTOPLASM FROM NITROGEN BALANCE

(Taken from Reifenstein Albright and Wells J Clin Endocrinol 5 367 95 Nov 1945)

COMPONENT TO BE DERIVED	FINAL UNIT	FACTOR
1 Protein in protoplasm	gm	6 5
2 Protoplasm fat free but not extracellular fluid free	gm	39
3 Protoplasm fat free and extracellular fluid free (true muscle)	gm	27
4 Intracellular fluid in protoplasm	ml	19
5 Extracellular fluid in protoplasm	ml	5
6 Potassium in intracellular fluid in protoplasm	mEq	2 7
7 Sodium in extracellular fluid in protoplasm	mEq	0 77
8 Phosphorus in protoplasm	gm	0 066
9 Sulfur in protoplasm	gm	0 069
10 Fat calorically equivalent to protoplasm	gm	9
11 Protoplasm (fat free) minus fat calorically equivalent to protoplasm	gm	9

To derive the component multiply nitrogen in grams by the factor

The paper of Reifenstein Albright and Wells gives the justification refinements and applications of this sort of theoretical calculation Their method of computation is different from that presented above To start their computation they calculate what the observed weight loss would have been had the subject been in nitrogen balance then express this in terms of fat and correct this fat figure for protoplasm (from Nitrogen balance) extracellular fluid (from Sodium balance) and intracellular fluid (from Potassium balance) Their method of calculation therefore involves one more assumption than the one presented in detail above i e the assumption that it is permissible to take the observed change in body weight as a function of fat Their method of calculation has the advantage that it emphasizes discrepancies between observed and theoretical weight change whereas the method presented above derives fat by difference and therefore the theoretical always equals the observed change in weight

SECTION IX

CLINICAL LABORATORY PROCEDURES

A MISCELLANEOUS REFERENCES TO TEXTBOOKS

It is not the intention of this manual to reduplicate detailed instructions for clinical laboratory procedures which have been well described in many textbooks of clinical methods The selected list of references below will give the reader a satisfactory working library in which may be found details of most of the methods commonly in use In the sections which follow, the term standard methods" refers to methods described in one or the other of the selected references References to individual papers will be found in the text that follows this bibliography

Bray W E Synopsis of Clinical Laboratory Methods, ed. 4 St Louis 1950 The C V Mosby Company

Gradwohl, R B H Clinical Laboratory Methods and Diagnosis ed 4 St Louis 1948, The C V Mosby Company

Ham H and others Syllabus for Second Year Course in Laboratory Diagnosis Preliminary Edition, 1948 Dept of Medicine Harvard Medical School, Longwood Publishing Company

Hawk P B Oser B L, and Summerson W H Practical Physiological Chemistry ed. 12 Philadelphia Toronto 1947 The Blakiston Company

Kolmer, J A Clinical Diagnosis by Laboratory Examinations 1st Edition revised New York London 1944 D Appleton Century Co Inc

Levinson S. A., and McFate, R P Clinical Laboratory Diagnosis ed. 3 Philadelphia 1946 Lea and Febiger

Osgood E E A Textbook of Laboratory Diagnosis ed 3 Philadelphia Toronto, 1948 The Blakiston Company

Simmons J S and Gentzkow C J Laboratory Methods of the United States Army ed 5, Philadelphia 1944 Lea and Febiger

Stitt E R Clough P W and Clough M. C Practical Bacteriology Hematology and Animal Parasitology ed 9, Philadelphia 1945 The Blakiston Company

Todd J C and Sanford A W Clinical Diagnosis With Laboratory Methods ed 10 Philadelphia and London 1943 W B Saunders Company

U S War Department Methods for Laboratory Technicians Oct 1946, U S Government Printing Office

Wadsworth A B Standard Methods of the Division of Laboratories and Research of the New York State Department of Health ed 3 Baltimore 1947, Williams and Wilkins Company

Whitby L E H. and Britton C J C Disorders of the Blood ed 5, Philadelphia 1946 The Blakiston Company

Wintrobe M. M Clinical Hematology ed. 2 (thoroughly revised) Philadelphia 1947, Lea and Febiger

SECTION IX

CLINICAL LABORATORY PROCEDURES (Cont'd)

B HEMATOLOGY

1 Calculation of Mean Corpuscular Volume Mean Corpuscular Hemoglobin Content and Mean Corpuscular Hemoglobin Concentration

The calculation of mean corpuscular volume hemoglobin content and hemoglobin concentration has been popularized by Wintrobe These derived values are useful in the classification of anemias The data required for the computation are

1 Erythrocyte count millions/cu mm
2 Hematocrit ml packed red cells/100 ml blood
3 Hemoglobin concentration gm Hb/100 ml blood

The equations used are

1 Mean corpuscular volume cubic microns =

$$\frac{\text{Hematocrit} \times 10}{\text{Erythrocyte count}}$$

Mean corpuscular hemoglobin content micromicrograms =

$$\frac{\text{Hemoglobin concentration of blood} \times 10}{\text{Erythrocyte count}}$$

3 Mean cell hemoglobin concentration % Hb =

$$\frac{\text{Hemoglobin concentration} \times 100}{\text{Hematocrit}}$$

The line chart (Fig 63) facilitates these computations

1 To obtain mean corpuscular volume stretch a straight edge between hematocrit (right hand) and red count (left hand) Read MCV on middle line
2 To obtain mean corpuscular hemoglobin content stretch a straight edge between hemoglobin concentration (right hand) and red count (left hand) Read MCH on middle line
3 To obtain mean corpuscular hemoglobin concentration stretch a straight edge between hemoglobin concentration (right hand) and hematocrit (left hand)

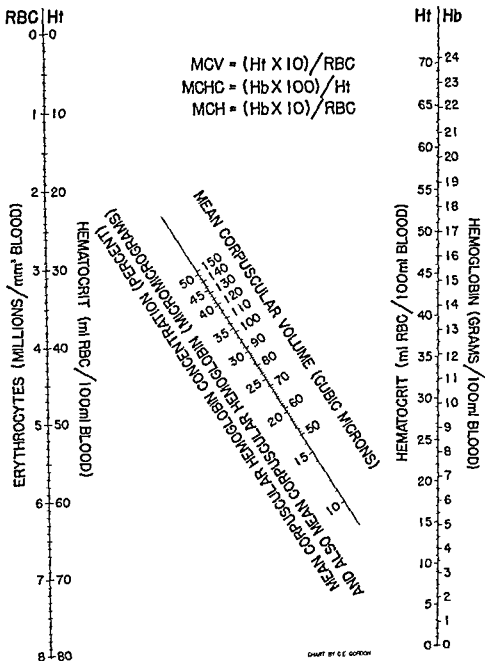


Fig 63 —Line chart for calculating cellular derived data using Wintrobe's equations.

2 Calculation of Sedimentation Rate

References

1 Rourke M D and Ernstene A. C A Method for Correcting the Erythrocyte Sedimentation Rate for Variations in the Cell Volume Percentage of Blood *J Clin Investigation* 8 545 559 (June) 1930

2 Wintrobe M M and Landsberg J W A Standardized Technique for the Blood Sedimentation Test Am J M S 189 102 115 (Jan) 1935

Calculation of Sedimentation Rate

Most authors who have published data on the blood sedimentation agree that it is necessary to convert the readings obtained to a standard hematocrit of 45 to 47 ml/100 ml blood Opinions vary on the exact form of the correction but it is agreed that the sedimentation rate becomes higher the lower the hematocrit The chart of Rourke and Ernstene will suffice for most of the methods

1 Obtain the uncorrected reading in mm per hour or mm per minute
2 Determine the hematocrit
3 On the chart (Fig 64) use the left hand line in mm per minute as it stands or multiply by 60 to convert to mm per hour
4 On the line for hematocrit as observed find the point for mm per minute as observed
5 Follow the empirical line down to hematocrit 45% and read the corrected sedimentation rate on the left hand line
6 Compare with the normal for hematocrit 45 (solid block)

3 Direct Eosinophil Count

Reference

Randolph T G Blood Studies in Allergy I. The Direct Counting Chamber Determinations of Eosinophils by Propylene Glycol Aqueous Stains J Allergy 15 89 96 (March) 1944

Randolph T G Differentiation and Enumeration of Eosinophils in the Counting Chamber With a Glycol Stain a Valuable Technique in Appraising ACTH Dosage J Lab & Clin Med 34 1696 1698 (Dec) 1949

Principle

Counting eosinophils from a differential smear is not accurate enough for many research purposes A direct count can be made in a counting chamber after dilution of the blood with the appropriate solution

Apparatus

1 A quantity of white blood cell pipettes
2 Counting chamber (Levy)
3 A microscope
4 Petri dishes
5 Mechanical shaker for blood pipettes

Reagents

1 Heparin solution Prepare heparin tubes by placing one drop of heparin solution in a glass vial of 10 ml capacity Allow drop to evaporate to dryness before using tube

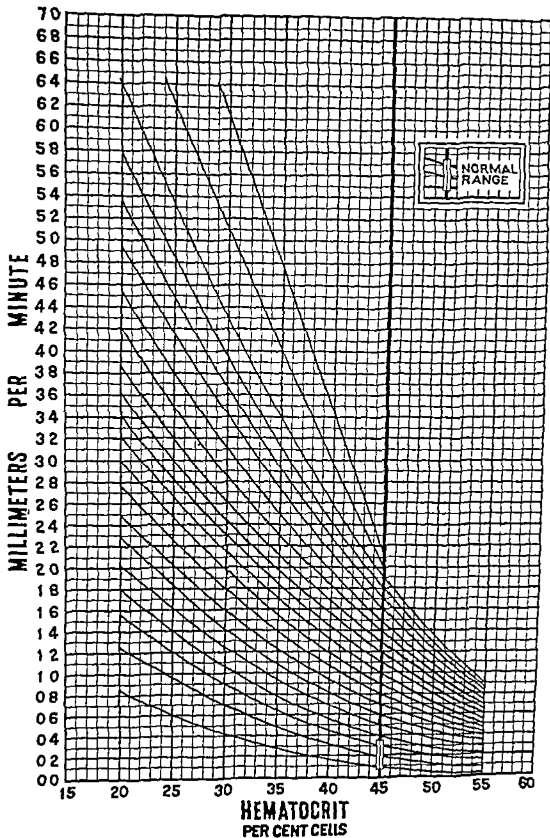


Fig 64 —Correction of hematocrit values by the method of Rourke and Ernstene.

2 D luting fluid
Solution A Prepare a 0 05 per cent solution of phloxin in propylene glycol and water (1 1 mixture) Stor in a brown bottle
Solution B Prepare an 0 05 per cent solution of methylene blue in propylene glycol and water (1 1 mixture) Store in a brown bottle
The diluting fluid is prepared fresh daily by mixing three parts of solution A with one pa t of solution B

Procedure

1 Draw 2 ml of blood by venipuncture and transfer the blood carefully to the heparinized tube M x gently
2 Af er thoroughly mixing draw blood to the 1 0 mark in a white blood count pipette
3 Bring the liquid level up to the 11 mark with the freshly prepared diluting fluid
4 Place the p pette in a mechan cal mix ng dev ce and mix the cell suspension for 0 minutes
5 After d scarding the first 4 to 5 drops fill two Levy counting chambers with flu d from the pipette
6 The filled counting chambers are placed under an inverted Petri dish containing a piece of mo st filter paper to prevent evaporat on Twenty to 30 minutes are allowed for the settl ng of cells
7 After sed mentation enumerate the eosinophils in the 9 square mill meters on both sides of the two counting chambers The eos nophils are identified by their deeply red stained granules Other granul s take a faint blue stain

Calculation

Total eosinophil count = average eosinophils enumerated per side (9 sq mm) × 11 = cells per cubic millimeter

Precautions

1 Timing is important Try to arrange the work so that all counts are made at ap prox mately the same time after d lution of the blood
2 The exact proportions of the diluting fluid are important

4 Hematology Miscellaneous References

Anticoagulants

Wintrobe M M J Lab & Clin Med 17 899 91 (June) 193
Moore B M Blood Coagulation and Its Clinical Modification With Anticoagu ants Texas Rep on Biol and Med 5 ( ) 1 31 1947

Ashby Count

Ebert R V and Emer on C P Jr A Clin cal Study of Transfusion Reactions The Hemolytic Effect of Group O Blood and Pooled Plasma Containing Incompatible Iso agglut nins J Clin Investigation 25 6 7 638 (July) 1946

Bone Marrow

Berman L and Axel od A R Aspirat on of Sternal Bone Marrow Techn c for Ob taining Volumetri Readings Smears Imprints and Histopathologic Sections Am J Cl n Path. 17 61 56 (Jan) 1947

Clotting Time

Pohle F J and Taylor F H. L Coagulat on Defect in Hemoph lia J Clin Investiga tion 16 741 747 (Sept) 1937

Erythrocytes

Jonx s J H P Determination of Red Blood Corpuscl s W th the Aid of a Photoelectric Colorimeter Nederl tijdschr v geneesk 92 20 4 036 1948
Pijper A The Diffraction Method of Measuring Red Llood Cells J Lab & Clin Med. 32 (7) 857 877 (July) 1947

Fibrinolysis

Tagren, H J Levenson S M Davidson, C S and Taylor F H L Occurrence of Fibrinolysis in Shock, With Observations on Prothrombin Time and Plasma Fibrinogen During Hemorrhagic Shock Am J M Sc 211 68 96 (Jan) 1946

Icteric Index

Hosley R J Nomograph for Rapid Calculation of Icterus Index When Determined by Colorimeter Am J Clin Path 19 884 885 (Sept) 1949

Leukocytes

Minor A H, and Burnett L A Method for Obtaining Living Leukocytes From Human Peripheral Blood by Acceleration of Erythrocyte Sedimentation Blood 3 799 802 (July) 1948

Vallee B L, Hughes W L Jr and Gibson, J G II The Separation of Leucocytes From Whole Blood by Flotation on Serum Albumin, Blood Special Issue 1 8 87 (July) 1947

Platelets

Pohle, F J The Blood Platelet Count in Relation to the Menstrual Cycle in Normal Women Am J M Sc 197 40 47 (Jan) 1939

Prothrombin Time

Munro F L and Munro M P The Preparation of Prothrombin by Adsorption on and Elution From Aluminum Hydroxide Arch Biochem 15 (2) 295 304 (Nov) 1947

Honorato René A Simple Means to Determine Exact Moment of Clotting in Prothrombin or Thrombin Time Determinations, Proc Soc Exper Biol & Med 65 (1) 41 (May) 1947

Ware Arnold G Guest M Mason and Seegers Walter H A Factor in Plasma Which Accelerates the Activation of Prothrombin J Biol Chem 169 (1) 231 232 (June) 1947

Sternberger L A The Stabilisation of Thrombin in Plasma Development of a Simple Two stage Method for the Determination of Prothrombin Brit J Exper Path 28 (3) 168 177 (June) 1947

SECTION IX

CLINICAL LABORATORY PROCEDURES (Cont'd)

C TOLERANCE TESTS

1 Gluco e Tolerance Tests

A. ORAL GLUCOSE TOLERANCE

Reference

Janney N W and Isaacson V I A Blood Sugar Tolerance Test Proc Soc Exper B ol & Med 15 1o 16 1917

Principle

After an oral dose of 50 to 75 gm of glucose the blood sugar concentration nor mally comes back to the control level in two hour and little if any glucose ap pears in the urine In diabetes mellit is the level stays high and gl icose appears in the urine In renal glycosuria the blood level behaves normally but glucose ap pears n the urine

Apparatus

1 Micro pipettes 0 1 ml a curately calibrated
2 A quantity of watch glasses
3 A quantity of 15 ml round bottom centr fuge tubes
4 Apparatus as for blood sugar analysis
5 A lan et for getting blood

Reagents

1 Dilute tungstic acid solution for deproteinizing blood (see glucose method)
2 Reagents for quantitative estimation of gluco e
3 Benedict s qualitative glucose reagent Weigh out separately 8 , gm copper sulfate ($CuSO_4$ $5H_2O$) 85 0 gm sod um citrate and 50 0 gm sodium carbonate (Na_2CO_3 anhydrous) D ssolve the citrate and carbonate in 400 ml of water and filter into a large beaker Dissolve copper sulfate n 50 ml of water and pour through a funn l into the bottom of the citrate carbonate solution As much as possible avo d agitation and loss of CO_2 to the air Dilute to one liter The solution keeps well at room temperature
4 Glucose solution The do e is 1 0 gm glucose/kg body we ght Dissolve glucose in 4 parts of water ch ll and flavor with l mon

Procedure

1 The subje t comes to the l bo atory without any breakfast Measure the body weight
   A blood sample is drawn from the ear or finger and 0 1 ml is diluted to 10 ml with ac d tungstate solut on
3 Collect a urine spec men
4 The subject then drinks the glucose mixtures as soon as poss ble and time is noted (If the mixture is cold it is more palatable)

5 Blood samples are then collected at 30 minutes 60 minutes 120 minutes and 180 minutes and analyzed for glucose by one of the quantitative methods

6 Urine samples are collected at one, two and three hours A qualitative test is run using Benedict s reagent Heat 10 ml of Benedict s reagent in a water bath. Add 8 drops of urine Heat for 5 minutes Cool Read in terms of color of sediment and supernatant fluid The gradations are blue green, yellow and brown

Calculation

As for glucose estimation

Precautions

1 As for glucose estimation

2 For interpretation see standard textbooks According to Conn (Conn J W Am J M Sc 199 555 564 1940) a short period of carbohydrate restriction causes a marked delay in the utilization of carbohydrate as indicated by the glucose tol erance test Undernourished persons lose even more carbohydrate tolerance than do normals Erroneous diagnosis of diabetes mellitus may be made when the factor of previous carbohydrate restriction is neglected Hence the use of preparatory diet (300 gm of carbohydrate 80 gm protein at best maintenance caloric intake) for at least 3 days before the test is recommended.

B INTRAVENOUS GLUCOSE TOLERANCE

Reference

Lozner E L Winkler A W Taylor F H L and Peters J P Intravenous Glucose Tolerance Test J Clin Investigation 20 507 515 (Sept) 1941

Principle

A single sugar determination on a specimen of blood drawn two hours after intra venous injection of 5 gm of glucose is a reliable guide in distinguishing between benign glycosuria and diabetes mellitus

Apparatus

1 Sterile needles and syringes for injection of glucose and drawing of blood

2 Apparatus as for glucose analysis

Reagents

1 Glucose solution a 50% sterile solution in water

2 Reagents as for blood sugar

Procedure

1 Prepare the patient as for oral glucose tolerance

2 Collect a sample of blood before injection

3 Inject 50 ml of glucose solution over a period of 10 minutes

4 For screening purposes a sample of blood at 2 hours is used For more precise studies samples are taken at 15 minutes 30 minutes 60 minutes and 120 min utes together with specimens of urine for qualitative test

Calculation, Precautions

Same as for oral test

Interpretation

See reference of Lozner and associates

2 Vitamin Tolerance Tests

A. ORAL VITAMIN TOLERANCE

References

1 Johnson P E Contreras L A Consolazio C F and Robinson P F A Comparison of Intravenous and Oral Vitamin Tolerance Tests Am J Physiol 144 58-68 (June) 1945

2 Goldsmith G A Chemical Measurements in Relation to Physical Evidence of Malnutrition Federation Proc 8 553 567 (June) 1949

Principle

A dose of vitamins is administered and the urine is collected thereafter for four hours If the body is well saturated with vitamins a considerable percentage of the test dose is excreted. If the body is unsaturated the vitamins are absorbed by the tissues and the urinary excretion is small

Apparatus (assuming 50 subjects)

1 A two gallon coffee pot or other container from which pouring is easy
2 A large spoon for stirring
3 50 paper cups
4. Apparatus for collecting and storing urine as described in previous sections

Reagents

1 Water soluble tablets of thiamine hydrochloride mg each
2 Water soluble tablets of riboflavin 5 mg each
3 Water soluble tablets of nicotinamide 50 mg each
4 Water soluble tablets of ascorbic acid 100 mg each
5 Chemicals required for collecting and storing urine

Procedure

1 Allow at least 2 hours for mixing the solutions before administration of the dose
2 For each subject expected add 150 ml of water to the pot and 1 tablet each of thiamine riboflavin and nicotinamide Stir until dissolved.
3 Before administration add for each subject 5 tablets of vitamin C Stir until dissolved Add the pills just in time to insure complete solution ½ hour is usually enough except in cold weather
4 On a convenient table place one cup for each subject and pour 150 ml of solution It is well to calibrate the cups with a heavy pencil line at the 150 ml mark
5 Immediately after the voiding of the fasting specimen command the subjects to file past the table take a cup drink the contents in the presence of an observer, and deposit the empty cups in a garbage can or other disposal unit provided.
6 The doses of vitamins are so large that the subject may now be allowed to eat breakfast without interfering with the test
7 All subjects should be briefed with instructions that the urine for the next 4 hours is to be collected If they have to urinate an observer should provide a paper cup for the purpose
8 Exactly 4 hours after the administration of the vitamins the specimens of urine should be collected measured and stored as described in section on storage of specimens

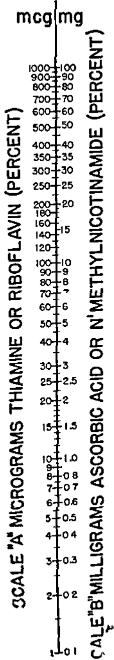
Calculations

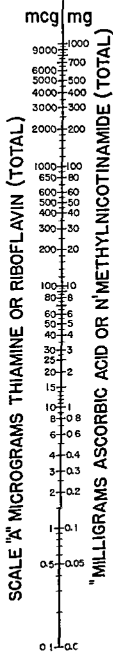
It is desired to determine how much of the dose of vitamins is excreted in 4 hours over and above the amount which would have been excreted if the subject had not taken any vitamins The assumption is made that the hourly excretion measured

EXCRETION OF VITAMINS FROM URINARY VOLUME AND CONCENTRATION OF VITAMINS

CONCENTRATION PER 100 ml URINE

TOTAL AMOUNT OF SUBSTANCE





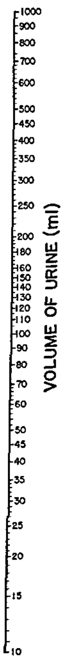


Fig 65

vitamins

in the fasting specimen would have changed very little if the subjects had not taken vitamins The following general formula is used

Excess excretion in 4 hours = ($\frac{\text{urine volume in load period}}{100}$ × con entration of vitamin in loaded specimen) − (excretion per hour in fasting specimen × 4)

Calculat ons are facilitated by the line chart in Fig 6

Example

1 Fasting urine

V tamin C	mg/hr	1	mg/4 hr	5
Vitamin B,	mcg/hr	12	mcg/4 hr	48
N[1] Methylnicotinamide	mg/hr	0 85	mg/4 hr	3 4
Vitamin B	m g/hr	80	mcg/4 hr	0

2 Tolerance test

Volume of urine in 4 hrs 300 ml

C	mg%	15	mg/4 hr	45	mg over fast ng	40
B	mcg%	80	m g/4 hr	200	mcg over fasting	42
NMN	mg%	2 0	mg/4 hr	7 2	mg over fasting	3 8
B	mcg%	500	m g/4 hr	1800	mcg over fasting	1480

Precautions

1 The solution of vitamins must be prote ted from direct sunlight at all times beca ise of the sens tivity of riboflavin

Vitamin C is so unstable that it must be added only just before administration

3 Very strict supervision is required to insure that the subjects drink all the vitamins and also that they collect all of their urine

4 The load test de cribed above is a necessary compromise between practical con ditions in th field and scientific desirability In particular a longer collection period would be des rable owing to the relatively slow excretion of certain of the vitamins Many workers feel that intravenous inje tion is preferable to oral adm nistration.

5 It is advisable to buy vitamins in large batches and essential to assay each batch before use

6 For every 50 subjects t is a good dea to make up enough solution for 5 extra doses n case of sp llage or inaccurate measuring

B INTRAVENOUS VITAMIN TOLERANCE

Reference

Same as for oral vitamin tolerance

Principle

Same as for oral vitamin tolerance

Apparatus

1 Sterile needles and syringes for inj cting v tamins
2 Apparatus for collect on and storage of urine spe imens

Reagents

1 Reagents for collecting and s oring urine
2 Ampoules of vitam n solutions for intravenous injection Because of incom pat bility of some of the vitamins it is necessary to have two sets of ampoules
   a Ascorbic acid 0 mg in 4 ml

b In 5 ml 5 mg thiamine hydrochloride 3 mg riboflavin, 75 mg nicotinamide 1 mg calcium pantothenate 1 mg of methylglucamine salt of folic acid 10 mg of biotin and 10 mg of pyridoxine hydrochloride

Procedure

1 Obtain a fasting hour specimen as described under oral vitamin tolerance
2 In a 20 ml sterile syringe mix 4 ml of the ascorbic acid with 5 ml of B complex solution and inject the 9 ml quantitatively

Calculation

Same as for oral vitamin tolerance

Precautions

Vitamin tolerance tests are not well standardized Their interpretation is well discussed in Bulletin No 117 National Research Council Nutrition Surveys Their Techniques and Value May 1949 A mathematical analysis of the time course of vitamin excretion is given by Johnson and colleagues

SECTION IX

CLINICAL LABORATORY PROCEDURES (Cont'd)

D KIDNEY FUNCTION

1 Titratable Acidity

Reference

Henderson L. J, and Palmer W W On Several Fa tors of Acid Excretion J Biol. Chem 17 305 315 (Apr) 1914.

Principle

Titratable acidity is measured by the amount of alkali necessary to alter the pH of a biolog cal fluid to 7 4

Apparatus

1 A quantity of 500 ml Erlenmeyer flasks and a burette calibrated in 0 0₅ ml divisions
2 Calibrated pipettes or syringe pipettes 2 and 10 ml
3 A 250 ml volumetric flask.

Reagents

1 Phosphate solution of pH 7 4 M/15 buffer solution prepared according to Søren sen s table on phosphate buffers Use 80 8 ml of M/15 Na_2HPO and 19 2 ml of M/15 KH_2PO_4
2 Sodium hydrox de (NaOH) a 0 1000 N *solution accurately titrated*
3 Phenol red 0 1% aqueous solution of sodium salt Dissolve 0.5 gm phenol red *in 500 ml of water and add 8 5 ml of 0 05 N NaOH*
4. Standard comparison solution In a " 0 ml volumetric flask dilute 2 ml phenol red and 10 ml standard phosphate buffer to 0 ml with water

Procedure

1 *To a 50 ml Erlenmeyer flask add exactly 10 ml of urine*
Add approx mately 150 ml of water and exactly 2 ml of phenol red solution
3 Dilute to the mark and shake well
4. Titrate the unknown with 0 1000 N NaOH until the color matches the standard. The end point may *be checked with the spectrophotom er at a wave length of* 511 mμ or the pH itself can be checked w th a glass electrode

Calculation

Titratable acidity ml of 1 N titratable acid/liter of urine =

$$\frac{(\text{ml alkali}) \times \text{normality of alkali} \times 1000}{\text{ml urine}}$$

Example

Ten ml of ur ne required 5 ₅ ml of 0 1000 N NaOH to change the pH to 7 4
Titratable acidity ml 1 N titratable acid/liter urine =

$$\frac{(5\quad) \times 0\,1000 \times 1000}{10} = 55\,5$$

Precautions

Matching the end point colors may be difficult in the presence of a highly pigmented specimen of urine In such cases it may be necessary to use a 5 ml sample

2 Phenolsulfonphthalein Test

Reference

Rowntree L G and Geraghty J T The Phthalein Test An Experimental and Clinical Study of Phenolsulphonphthalein in Relation to Renal Function in Health and Disease Arch Int Med 9 284 388 (March) 1912

Principle

The normal kidney clears injected phenolsulfonphthalein rapidly from the blood stream The amount of dye excreted in a given time is measured spectrophotometrically

Apparatus

1 Graduated cylinders 1000 ml
2 Coleman Jr Spectrophotometer
3 Cuvettes 19 × 150 mm.
4 Sterile 1 ml tuberculin syringes and #23 needles

Reagents

1 Sodium hydroxide approximately 1 N 40 gm/1000 ml water
2 Phenolsulfonphthalein (PSP) 1 ml equivalent to 6 mg of dye sterilized in ampoules
3 Standard solution of dye Dilute 1 ml of dye (= 6 *mg dye*) *to exactly 2000 ml* with water containing a few ml of NaOH

Procedure—Collection of Specimens

1 The subject omits breakfast the morning of the test
2 Allow the subject to drink 300 400 ml of water
3 One half hour later instruct him to urinate and discard the specimen
4 Inject 1 ml of PSP (6 mg) intravenously and note the time
5 Collect urine samples quantitatively at 15 30 and 60 minutes

Procedure—Analysis of Specimens

1 Pour the whole specimen of urine into a graduated liter cylinder
2 Add enough NaOH to make urine alkaline (10 15 ml)
3 Dilute to 1000 ml Mix well
4 Read aliquot in cuvette setting water blank at 100% T at 520 mμ

Calibration Curve

1 Using the standard PSP solution as described in reagents prepare a calibration curve of the following dilutions

STANDARD ml	WATER ml	DYE/l mg	% OF DYE INJECTED
100	0	3 0	50
80	20	2 4	40
60	40	1 8	30
40	60	1 2	20
20	80	0 6	10
10	90	0 3	5

2 Read these against a water blank set at 100% T at wave length 520 mμ.

3 Plot the calibration curve of % T against % dye excreted

Calculation

1 Calculate % dye in each specimen by reference to the calibration curve
2 % dye excreted in 60 minutes =
(Sum of % dye in specimens 1 and 3)

Example

1 The % dye excreted in specimen 1 was 45 in specimen 25 and in specimen 10
2 % dye excreted in 60 minutes =
45 + 25 + 10 = 80%
3 The time course of excretion was normal

Precautions

1 New calibration curves should be run with every new batch of dye
2 In heavily pigmented urine, a urine blank instead of a water blank may be required.

3 Renal Clearance Tests Miscellaneous References

General

Smith H W The Physiology of the Kidney New York 1937, Oxford University Press
Smith H W Notes on the Interpretation of Clearance Methods J Clin Investigation 20 631 635 (Nov) 1941

Diodrast

White H L and Rolf D A Rapid Micro Method for Determining Diodrast and Inorganic Iodide Iodine in Blood and Urine Proc Soc Exper Biol & Med 43 1 (Jan) 1940

Glomerular Filtration Rate

Earle D P Jr and Berliner R W A Simplified Clinical Procedure for Measurement of Glomerular Filtration Rate and Renal Plasma Flow Proc Soc Exper Biol & Med 62 (2) 62 64 (June) 1946

Inulin Clearance

Shannon J A and Smith H W The Excretion of Inulin Xylose and Urea by Normal Man J Clin Investigation 14 393 401 (July) 1935

Phenolsulphonphthalein Test

Scardino P L, and Scott W W The Determination of Phenolsulphonphthalein With the Photoelectric Colorimeter and Its Application of the Phthalein Elimination Curve J Urology 58 (2) 143 150 (Aug) 1947

Renal Blood Flow

Foà P P and Foà N L A Simple Method for Determining Effective Renal Blood Flow and Tubular Excretory Mass in Man Proc Soc Exper Biol & Med 51 (3) 3 3 8 (Dec) 194

Urea Clearance

Peters J P and Van Slyke D D Quantitative Clinical Chemistry Vol II Methods Baltimore 19 Williams and Wilkins Company pages 564 572

SECTION IX

CLINICAL LABORATORY PROCEDURES (Cont'd)

E LIVER FUNCTION

1 Bromsulfalein Test

References

1 Rosenthal S M and White E C Clinical Application of the Bromsulphalein Test for Hepatic Function J A M A 84 1112 1114 (April) 1925
2 Mateer G Baltz J I Marion D F, and MacMillan, J M Liver Function Tests J A M A 121 723 728 (March) 1943
3 Gaebler O H Determination of Bromsulfalein in Normal Turbid Hemolyzed or Icteric Serums *Am J Clin Path 15 452 455 (Oct) 1945*

Principle

The dye bromsulfalein is removed from the blood stream by the liver After intra venous injection bromsulfalein in the serum is measured colorimetrically

Reagents

1 Bromsulphalein dye 150 mg/3 ml ampoule
2 Hydrochloric acid a 5% solution
3 Sodium hydroxide, 0 1 N solution, 4 gm/1000 ml of water

Apparatus

1 Coleman Jr Spectrophotometer Model 6
2 Cuvettes 19 × 150 mm
3 A quantity of 15 ml centrifuge tubes (round bottom)
4 Electric centrifuge
5 Syringes 5 ml and #20 gauge needles (both sterile)
6 Syringe pipette 1 0 ml

Procedure—Collection of Specimens

1 The patient is weighed and the dosage to be injected is calculated on a basis of 5 mg per kilogram of body weight
2 The calculated amount of dye to be injected is measured into a 5 ml syringe
3 A control specimen is drawn from a vein. The syringe is separated from the *needle and the syringe containing the dye is connected in its place* The dye is slowly injected into the vein the time being approximately one minute *Care should be taken* not to allow infiltration of the dye outside the vein, as it is very painful
4 Record the time of injection and after 45 minutes draw a sample of blood (4 to 5 ml) preferably from the other arm. Allow the blood to run into a clean dry test tube
5 Allow the blood to coagulate and then centrifuge and separate the serum

Procedure—Analysis of Serum

1 Into each of two cuvettes pipette exactly 0 5 ml serum
2 To the control tube add 2 5 ml water and 3 ml of hydrochloric acid. Mix.

3 To the other tube add 2 . ml of water and 3 ml of 0 1 N sodium hydroxide
4 Set the control at 100% T at 565 mμ and read the unknown

Calibration Curve

1 Make up dilution of the dye in dilute sodium hydroxide in the range 0 to 0 1 mg/6 ml
^ Using a water blank set at 100% T read the standards at a wave length of 565 mμ.

Calculation

1 A value of 10 mg bromsulfalein/100 ml of serum is arbitrarily taken as 100% retention when the dosage is 5 mg of bromsulfalein/kilogram body weight

$$\% \text{ retention} = \frac{(\text{mg in cuvette}) \times 100 \times 100}{(\text{ml serum in cuvette}) \times 10}$$

Example

The % T was 91 % corresponding to 0 0 mg bromsulfalein in the cuvette
% retention =

$$\frac{(0\,0\ ) \times 100 \times 100}{0\,5 \times 10} = 40\%$$

Precautions

1 Painful sloughing will be caused by extravascular bromsulfalein

2 Serum Bilirubin

Reference

Malloy H T and Evelyn K. A. The Determination of B lirub n With the Photo electric Colorimeter J Biol. Chem 119 481 490 (July) 1937

Principle

Bilirubin like aromatic am nes combines with diazonium salts to form azo dyes Addition of alcohol sets all the bilirubin free to react w th the diazo reagent and is u ed to make the react on quant tative for total bil rubin content of the serum

Apparatus

1 Coleman Jr Spectrophotometer Model 6
2 Cuvettes 19 x 1.0 mm
3 A quantity of volumetric flasks 10 ml or ^5 ml
4 A 1 0 ml syringe p pette accurately calibrated

Reagents

1 Methyl alcohol absolute
2 Sulfanilic acid sol tion D solve 1 gm of sulfanilic acid n 15 ml of concentrated HCl and dilute to 1 liter with distilled water
3 Sodium nitrite solution Dissolve 0 5 gm of NaNO (ACS) in a little water and d lute to 100 ml The sol it on is to be prepared on the day it is used
4 Diazo reagent Add 0 3 ml of the nitrite solution to 10 ml of the sulfanilic acid solution. The diazo reagent must be prepared with n a few m nutes of the time when it is to be used
5 D lute HCl for blanks Dilute 15 ml of concentrated HCl to 1 liter with water
6 B lirub n stock standard. Transfer exactly 40 mg of purified b lirub n to a 100 ml glass stoppered volumetric flask. Dissolve bring to the mark with chloroform (USP) and mix by inverting 10 times (This solution is stable if stored in a brown bottle with properly fitted glass stopper)

Bilirubin working standard Dilute 1 ml of the above stock solution to 100 ml with absolute methyl alcohol The working standard is 0 4 mg/100 ml

Procedure—Collection of Specimens

1 To avoid turbidity of serum by alimentary lipemia the blood should be taken when the patient is in the postabsorptive state Transfer the sample to a round bottom tube without anticoagulant close with a pledget of cotton and then keep at room temperature for approximately 15 minutes until the clot is completely formed Chill in ice water and place in the refrigerator until the clot has contracted after which it is gently freed from the top part of the tube if there is adherence and the tube then centrifuged After centrifuging transfer the clear serum to a clean dry tube The diazo reaction must be applied within 2 hours after the blood is clotted and if the separated serum is allowed to stand the reaction is altered After long standing only the indirect reactions occur

Procedure—Analysis of Serum

1 Pipette exactly 1 ml of serum into a 10 ml volumetric flask fill to the mark with distilled water stopper and mix by inverting 10 times
2 Into a cuvette pipette exactly 5 ml of absolute methyl alcohol and then 1 ml of fresh diazo reagent
3 Follow with exactly 4 ml of the (9 + 1) diluted serum mix stopper and hold 30 minutes at room temperature for the full color to develop (If bilirubin exceeds 5 mg/100 ml dilute the serum to 25 ml instead of 10 ml)
4 At the same time prepare a reagent blank with 4 ml of dilute hydrochloric acid 5 ml of absolute methyl alcohol and 1 ml of diazo reagent
5 Set the blank at 100% T at 550 mμ and read the unknown

Calibration Curve

1 Prepare a calibration curve with working bilirubin standard in the range 0 to 0 016 mg bilirubin per cuvette Mix in each case 5 ml of absolute methyl alcohol 1 ml of diazo reagent the requisite amount of working standard and dilute hydrochloric acid to make the total final volume 10 ml
2 Stopper all cuvettes tightly mix each by inverting 4 times Let stand together for 30 minutes at room temperature for the color to develop fully then measure the Transmittance (T) at 550 mμ

Calculation

mg bilirubin/100 mg serum =

$$\frac{(\text{mg in cuvette}) \times (\text{dilution}) \times 100}{(\text{ml aliquot}) \times (\text{ml serum})}$$

Example

One ml serum was diluted to 10 ml of which 4 ml were taken for analysis A reading of 80% T was equivalent to 0 010 mg bilirubin in the cuvette
mg total bilirubin/100 ml serum =

$$\frac{(0.010) \times 10 \times 100}{4 \times 1} = 2.5 \text{ mg bilirubin}$$

Precautions

1 Careful handling of the serum is essential to this method
2 If it is desired to determine direct and indirect bilirubin two stages are required (a) without methyl alcohol (b) with methyl alcohol See the original paper for this procedure

3 Urobilinogen in Urine and Feces

A. UROBILINOGEN IN URINE

Reference

Schwartz S Sborov V and Watson C J Urobilinogen IV The Determination of Urobilinogen by Means of the Evelyn Photoelectric Colorimeter Am J Clin. Path 14 598 604 (Dec) 1944

Principle

A red color is formed by the reaction between urobilinogen and Ehrlich s aldehyde reagent

Apparatus

1 Separatory funnels 300 ml
  Graduated cylinders 100 ml
3 Cuvettes size 19 × 150 mm
4. Coleman Jr Spe trophotometer Model 6
5 A quant ty of 1‸5 ml Erlenmeyer flasks
6 An 0 5 ml syringe pipette accurately cal brated

Reagents

1 Sodium carbonate (reagent grade)
2 Petroleum eth r (b p 30 60 C)
3 Ferrous sulfate a 0% solution (freshly prepared) 0 gm of ferrous sulfate are dissolved in 9 ml of water
4. Sodium hydroxide a 10% solution in water
5 Ehrlich s aldehyde reagent 0 7 gm para dimethyl am ino benzaldehyde 150 ml of concentrated hydrochloric acid and 100 ml of water
6 Sodium acetate aqueous saturated solution
7 Glacial acetic acid
8 Ethyl alcohol 95%
9 Standard solution Dissolve 5 mg of Pontacyl Carm ne °B (Du Pont) and 95 mg of Pontacyl Violet 6R in 1000 ml of 0 5% acet c acid A further dilution of one part in 6 gives a color intensity equivalent to 0 6 mg of urobilinogen per 100 ml With a drop of CHCl the color is stable indefinitely (If available it is better to use pure crystalline urobilinogen)

Preservation of Sample

The procedure is applicable to an nd vidual o a 4 hour sample If the latter the sample should be in a dark bottle contain ng approx 5 gm of sodium carbonate under a layer of petroleum ether

Procedure

1 To 50 ml of urine in a 1 5 ml Erlenmeyer flask add 5 ml of ferrous sulfate solution
2 Add 5 ml of 10% sodium hydroxide mix thoroughly and set away in the dark for one hour Filter or centrifuge
3 At the end of the hour the filtrate should not exh bit any urobilin band at 08 mμ To determ ne the amount of filtrate to use do a qualitative test first
   a To ‸ ml of the filtrate add ‸ ml of Ehrl ch s reagent and 4 to 6 ml of sodium acetate solut on
   b If the resultant color is an intense red 0 5 ml of filtrate is enough for the quantitative procedure If pale red use 5 to 10 ml if faint pink use 15 to 5 ml and if there is no color use 0 ml.
4 The amount selected is placed in a pear shaped funnel and if small is diluted to bring the volume to ‸ ml
5 Then add 50 ml of petroleum ether and ac dify with 5 ml of glacial acetic ac d

6 Shake the mixture vigorously and allow to separate The emulsions are broken by the addition of more acetic acid and in some cases 1 2 ml of 95% ethyl alcohold is used
7 Decant the petroleum ether from the top by running out as much of the aqueous fraction as possible through the lower stopcock The aqueous layer is extracted twice more with 25 ml portions of petroleum ether
8 The combined ether extractions are placed in a clean separatory funnel and washed once with a small amount of distilled water which is discarded. This serves to remove small amounts of mechanically entrained impurities
9 Add 2 ml of Ehrlich s reagent and shake the solutions vigorously
10 Now add 6 ml of the sodium acetate solution and shake the mixture again (At times it is noted at this stage that the color of the aqueous fraction becomes somewhat less intense and at the same time a fine reddish brown precipitate appears at the interphase This is believed to be indol or skatol aldehyde since it has been observed repeatedly that the same phenomenon occurs when indol is similarly handled with petroleum ether Ehrlich s reagent and sodium acetate)
11 The colored aqueous fraction after complete separation is removed into a 100 ml graduated cylinder
12 Repeat the extraction of the petroleum ether with the Ehrlich s reagent and sodium acetate one or more times or until the final aqueous fraction is not more than very faint Dilute to 100 ml
13 Place 10 ml of the well mixed solution into a cuvette
14 For a blank reading add 9 ml of sodium acetate to 3 ml of Ehrlich's reagent. Set at 100% T at 508 mμ and read the unknown

Calibration Curve

The arbitrary standard is equivalent to 0 6 mg urobilinogen/100 ml Set up a calibration curve in the range from 0 to 10 mcg in 10 ml and run at 508 mμ

Calculation

mg urobilinogen/liter of urine =

$$\frac{(\text{mcg in cuvette}) \times (\text{final dilution}) \times (\text{initial dilution}) \times (1000)}{(1000) \times (\text{ml final aliquot}) \times (\text{ml filtrate}) \times (\text{ml urine})}$$

Example

A reading of 75% T was equivalent to 1 6 mcg in 10 ml in the cuvette Ten ml aliquots were read of a final dilution of 100 ml. Ten ml of filtrate had been analyzed from an initial dilution of 50 ml of urine to 100
mg urobilinogen/liter of urine =

$$\frac{(1\ 6) \times 100 \times 100 \times 1000}{1000 \times 10 \times 10 \times 50} = 3\ 2 \text{ mg/liter}$$

Precautions

1 There are many places in this method at which losses will occur Care is required at every step The method itself is not as quantitative as one would desire

B UROBILINOGEN IN FECES

Reference Principle

Same as for urobilinogen in urine

Apparatus

Same as for urobilinogen in urine and in addition

1 Quantitative filter paper
2 A number of funnels
3 A Waring Blendor

Reagents

Same as for urobilinogen in urine

Procedure—Processing of Sample

1 All fecal matter for a period of 3 days is collected as described in the section of this manual on collection of feces

2 In Waring Blendor the whole mass is homogenized and finally diluted to exactly 000 ml

Procedure—Preparation of the Alkaline Extract of Stool

1 Dilute 25 to 100 ml of the emulsion up to exactly 300 ml with water

2 Add 100 ml of ferrous sulfate

3 Then add 100 ml of sodium hydroxide stopper the flask shake and place in the dark for one to two hours

4 Filter through quantitative filter paper

Procedure—Analysis of Alkaline Extract

1 Pipette 50 ml of the extract into a separatory funnel

2 Add 1 ml glacial acetic acid

3 Then add 30 40 ml of petroleum ether and shake for three minutes

4 Release the watery part into a second separatory funnel add 30 40 ml petroleum ether again and shake for three minutes Combine both ether extracts

5 Wash twice with distilled water and then add 1 ml of Ehrlich s aldehyde reagent

6 Shake for one minute and then add 4 ml of a cold solution of sodium acetate (saturated)

7 Shake for 3 minutes and release the colored phase into a 100 ml graduated cylinder

8 Repeat the process of adding the Ehrlich s reagent and sodium acetate until no colored compound is formed.

9 Combine all the colored washings dilute to 100 ml and read an aliquot in the spectrophotometer setting the blank at 100% T as for urine

Calibration Curve

Same as for urobilinogen in urine

Calculation

mg urobilinogen in feces/day =

$$\frac{(\text{mcg in cuvette}) \times (\text{dilution } \# \ ) \times (\text{dilution } \#1) \times (2000)}{(1000) \times (\text{ml aliquot}) \quad (\text{ml alkaline filt at }) \quad (\text{ml stool}) \times (\text{days collected})}$$

Example

A 3 day sample was diluted to 000 ml Of the emulsion 50 ml were diluted to 500 of which 50 ml aliquots were analyzed the final dilution being to 100 ml. The concentration of urobilinogen in the cuvette was 15 mcg in 10 ml

mg fecal urobilinogen/day =

$$\frac{15 \times 100 \times 500 \times 000}{1000 \times 10 \times 0 \times 50 \times 3} = 50 \text{ mg/day}$$

Precautions

Same as for urobilinogen in urine

4 Liver Function, Miscellaneous References

General

Mateer, J G Baltz J L Steele H H Brouwer S W and Colvert J P Chronic Subclinical Impairment of the Liver Early Diagnosis and Treatment Further Improvements and Evaluation of Certain Liver Function Test J A M A 133 909 916 (March) 1947

Osgood Edwin E Interpretation of Liver Function Tests J A M A 134 (7) 585 83 (June) 1947

Amyloidosis

Unger P N Zuckerbrod M Beck G J and Steele J M Study of the Disappearance of Congo Red From the Blood of Non amyloid Subjects and Patients With Amyloidosis J Clin Investigation 27 111 117 (Jan) 1948

Bile in Urine

Hawkinson V, Watson C J and Turner R H Modification of Harrison s Test for Bilirubin in Urine Especially Suited for Mass and Serial Use J A M A 129 514 515 (Oct) 1945

Cephalin Flocculation

Hanger F M Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin Cholesterol Emulsion J Clin Investigation 18 61 69 (May) 1939

Cholinesterase Activity

Vorhaus L J II and Kark R M Measurement of Serum Cholinesterase Activity A Useful Laboratory Tool in the Study of Diseases of the Liver and Biliary System To be published Medical Nutrition Laboratory Report No 56 June 8 1949

Galactose

Colcher H Patek A J Jr and Kendall F E Galactose Disappearance From the Blood Stream Calculation of the Galactose Removal Constant and Its Application as a Test for Liver Function J Clin Investigation 25 768 775 (Sept) 1946

Hemosiderin in Urine

Rous P Urinary Siderosis Hemosiderin Granules in the Urine as an Aid to Diagnosis of Pernicious Anemia Hemachromatosis and Other *Diseases Causing Siderosis* J Exper Med 28 645 658 (Nov) 1918

Hippuric Acid

Quick A J The Clinical Application of the Hippuric Acid and Prothrombin Test Am J Clin Path 10 222 233 (March) 1940

Phosphatase

Gutman A B and Hanger F M Jr Differential Diagnosis of Jaundice by Combined Serum Phosphatase Determination and Cephalin Flocculation Test M Clin North America 25 837 848 (May) 1941

Portal Pressure

Bean W B Paul W D and Franklin M Preliminary Studies on an Indirect Method for Determining Portal Pressures J Clin Investigation 28 769 770 (July) 1949

Prothrombin Time

Aggeler P M Howard J Lucia S P Clark W and Astaff A Standardization of the Quick Prothrombin Test Blood 1 220 233 (March) 1946

Quick A J The Clinical Significance of Prothrombin as a Factor in Hemorrhage Pennsylvania M J 43 125 130 (Oct) 1939

Unger P N and Shapiro S Prothrombin Response to the Parenteral Administration of Large Doses of Vitamin K in Subjects With Normal Liver Function and in Cases of *Liver Disease A Standardized Test for the Estimation of Hepatic Function* J Clin Investigation 27 39 47 (Jan) 1948

Thymol Turbidity

Dekema H The Thymol Turbidity Test and the Cephalin Cholesterol Flocculation Test Nederl tijdschr v geneesk 93 (II) 19 4 8? (1949)

Huerga J de la and Popper H *Standardized Reagent for Thymol Turbidity Test* J Lab & Clin Med 34 (6) 877 880 (June) 1949

Shank R E and Hoagland C L A Modified Method for the Quantitative Determination of the Thymol Turbidity Reaction of Serum J Biol Chem 162 133 138 (Jan) 1946

Urobilinogen

Wilson T M and Davidson L S P Ehrlich s Aldehyde Test for Urobilinogen British M J 1949 884 889 (May) 1949

SECTION IX

CLINICAL LABORATORY PROCEDURES (Cont'd)

F FUNCTIONAL TESTS OF ENDOCRINE GASTRIC AND OTHER SYSTEMS

1 Pituitary Adrenocorticotrophic Hormone (ACTH) Test

Reference

Forsham P H Thorn G W Prunty F T G and Hills A G Clinical Studies With Pituitary Adrenocorticotrophin J Clin Endocrinol 8 15 66 (Jan) 1948

Principle

Following the injection of pituitary adrenocorticotrophic hormone (ACTH) in normal individuals there is an immediate and striking fall in circulating eosinophils and a rise in the uric acid excretion Patients with Addison s disease fail to show these changes

Apparatus

1 Sterile syringe and needle for intramuscular injection of ACTH
2 Apparatus for direct eosinophil count
3 Apparatus for estimating uric acid and creatinine

Reagents

1 ACTH for Intramuscular Injection The solubility varies with different batches of ACTH The hormone requires alkalinization to a pH between 8 and 9 (faint pink with phenolphthalein) To 5 ml of sterile saline add three drops of approximately N/10 NaOH This is taken up in a syringe and added to the rubber capped vial containing 25 mg of ACTH Shake gently until homogeneous A somewhat cloudy solution is obtained This should not be kept longer than twelve hours at 4 C or longer than two hours at room temperature It should be frozen for long storage The powder appears stable
2 Reagents for direct eosinophil count
Reagents for estimating uric acid and creatinine

Procedure

1 No food is allowed after 6 00 P M at night
2 On the following day give the patient 200 ml of water at 6 00 8 00 and 10 00 A M
3 Collect urine from 6 00 to 8 00 A M
4 An eosinophil count is done at 8 00 A M
5 Immediately after the 8 00 A M specimen inject 25 mg ACTH intramuscularly deep into the gluteus maximus A moderate degree of soreness often persists for a day or two
6 Urine is collected from 8 00 A M to 12 00 noon and the eosinophil count is repeated at noon
7 Analyze the urine specimens for uric acid and creatinine

Amyloidosis

Unger P N Zuckerbrod M Beck G J and Steele J M Study of the Disappearance of Congo Red From the Blood of Non amyloid Subjects and Patients With Amyloidosis J Clin Investigation 27 111 117 (Jan) 1948

Bile in Urine

Hawkinson V Watson C J and Turner R H Modification of Harrison s Test for Bilirubin in Urine Epecially Suited for Mass and Serial Use J A M A 129 514 515 (Oct) 1945

Cephalin Flocculation

Hanger F M Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin Cholesterol Emulsion J Clin Investigation 18 261 69 (May) 1939

Cholinesterase Activity

Vorhaus L J II and Kark R M Measurement of Serum Cholinesterase Activity A Useful Laboratory Tool in the Study of Di ea es of the Liver and Biliary System To be published Med cal Nutrition Laboratory Report No 56 June 8 1949

Galactose

Colcher H Patek A J Jr and Kendall F E Galactose Di appearance From the Blood Stream Calculation of the Galacto e Removal Constant and Its Application as a Test for Liver Function J Clin Investigation 25 768 75 (Sept) 1946

Hemosiderin in Urine

Rous P Urinary Siderosis Hemo iderin Granules in the Urine as an Aid to Diagnosis of Pernicious Anemia Hemachromatosis and Other Diseases Causing Siderosis J Exper Med 28 645 658 (Nov) 1918

Hippuric Acid

Quick A J The Clinical Application of the Hippuric Acid and Prothrombin Test Am J Clin Path 10 2 2 293 (March) 1940

Phosphatase

Gutman A B and Hanger F M Jr Differential Diagno is of Jaundice by Combin d Serum Phosphatase Determination and Cephalin Flocculation Te t M Clin North America 25 83 848 (May) 1941

Portal Pressure

Bean W B Paul W D and Franklin M Preliminar Studies on an Indirect Method for Determining Portal Pressures J Clin Investigation 28 769 770 (July) 1949

Prothrombin Time

Aggeler P M Howard J, Lucia S P Clark W and Astaff A Standardization of the Quick Prothrombin Test Blood 1 220 233 (March) 1946

Quick A J The Clinical Significance of Prothrombin as a Factor in Hemorrhage Pennsylvania M J 43 125 130 (Oct) 1939

Unger P N and Shapiro S Prothrombin Re ponse to the Parenteral Administration of Large Do es of Vitamin K in Subjects With Normal Liver Function and in Cases of Liver Disea e A Standardized Te t for the Estimation of Hepatic Function J Clin Investigation 27 39 47 (Jan) 1948

Thymol Turbidity

Dekema H The Thymol Turbidity Test and the Cephalin Cholesterol Flocculation Test Nederl tijd chr v geneesk 93 (II) 19 4 82 (1949)

Huerga J dela and Popper H Standardized Reagent for Thymol Turbidity Test J Lab & Clin Med 34 (6) 87 880 (June) 1949

Shank R E and Hoagland C L A Modified Method for the Quantitative Determination of the Thymol Turbidity Reaction of Serum J Biol Chem 162 133 138 (Jan) 1946

Urobilinogen

Wilson T M and Davidson L S P Ehrlich s Aldehyde Test for Urobilinogen British M J 1949 884 889 (May) 1949

SECTION IX

CLINICAL LABORATORY PROCEDURES (Cont'd)

F FUNCTIONAL TESTS OF ENDOCRINE GASTRIC AND OTHER SYSTEMS

1 Pituitary Adrenocorticotrophic Hormone (ACTH) Test

Reference

Forsham P H Thorn G W Prunty F T G and Hills A G Clinical Studies With Pituitary Adrenocorticotrophin J Clin Endocrinol 8 15 66 (Jan) 1948

Principle

Following the injection of pituitary adrenocorticotrophic hormone (ACTH) in normal individuals there is an immediate and striking fall in circulating eosinophils and a rise in the uric acid excretion Patients with Addison s disease fail to show these changes

Apparatus

1 Sterile syringe and needle for intramuscular injection of ACTH
2 Apparatus for direct eosinophil count
3 Apparatus for estimating uric acid and creatinine

Reagents

1 ACTH for Intramuscular Injection The solubility varies with different batches of ACTH The hormone requires alkalinization to a pH between 8 and 9 (faint pink with phenolphthalein) To 5 ml of sterile saline add three drops of approximately N/10 NaOH This is taken up in a syringe and added to the rubber capped vial containing 25 mg of ACTH Shake gently until homogeneous A somewhat cloudy solution is obtained This should not be kept longer than twelve hours at 4 C or longer than two hours at room temperature It should be frozen for long storage The powder appears stable
2 Reagents for direct eosinophil count
3 Reagents for estimating uric acid and creatinine

Procedure

1 No food is allowed after 6 00 P M at night
2 On the following day give the patient 200 ml of water at 6 00 8 00 and 10 00 A M
3 Collect urine from 6 00 to 8 00 A M
4 An eosinophil count is done at 8 00 A M
5 Immediately after the 8 00 A M specimen inject 25 mg ACTH intramuscularly deep into the gluteus maximus A moderate degree of soreness often persists for a day or two
6 Urine is collected from 8 00 A.M to 12 00 noon, and the eosinophil count is repeated at noon
7 Analyze the urine specimens for uric acid and creatinine

Amyloidosis

Unger P N Zuckerbrod M Beck G J and Steele J M Study of the Disappearance of Congo Red From the Blood of Non amyloid Subjects and Patients With Amyloidosis J Clin Investigation 27 111 117 (Jan) 1948

Bile in Urine

Hawkinson V Watson C J and Turner R H Modification of Harrison s Test for Bilirubin in Urine Epecially Suited for Mass and Serial Use J A M A 129 514 515 (Oct) 1945

Cephalin Flocculation

Hanger F M Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin Cholesterol Emulsion J Clin Investigation 18 261 69 (May) 1939

Cholinesterase Activity

Vorhaus L J II and Kark R M Measurement of Serum Cholinesterase Activity A Useful Laboratory Tool in the Study of Diseases of the Liver and Biliary System To be publi hed Med cal Nutrition Laboratory Report No 56 June 8 1949

Galactose

Colcher H Patek A J Jr and Tendall F E Galactose Disappearance From the Blood Stream Calculation of the Galacto e Removal Constant and Its Application as a Test for Liver Function J Clin Investigation 25 768 75 (Sept) 1946

Hemosiderin in Urine

Rous P Urinary Sidero is Hemosiderin Granules in the Urine as an Aid to Diagnosis of Pernicious Anemia Hemachromatosis and Other Disea es Causing Siderosis J Exper Med 28 645 658 (Nov) 1918

Hippuric Acid

Quick A J The Clinical Application of the Hippuric Acid and Prothrombin Test Am J Clin Path 10 222 ° 3 (March) 1940

Phosphatase

Gutman A B and Hanger F M Jr Differential Diagno is of Jaundice by Combined Serum Phosphata e Determination and Cephalin Flocculation Test M Clin North America 25 837 848 (May) 1941

Portal Pressure

Bean W B Paul W D and Franklin M Preliminary Studies on an Indirect Method for Determining Portal Pressures J Clin Investigation 28 769 770 (July) 1949

Prothrombin Time

Aggeler P M Howard J Lucia S P Clark W and Astaff A Standardization of the Quick Prothrombin Test Blood 1 220 223 (March) 1946

Quick A J The Clinical Significance of Prothrombin as a Factor in Hemorrhage Pennsylvania M J 43 125 130 (Oct) 1939

Unger P N and Shapiro S Prothrombin Response to the Parenteral Admini tration of Large Doses of Vitamin K in Subjects With Normal Liver Function and in Ca es of Liver Disease A Standardi ed Test for the Estimation of Hepatic Function J Clin Investigation 27 39 47 (Jan) 1948

Thymol Turbidity

Dekema H The Thymol Turbidity Test and the Cephalin Cholesterol Flocculation Test Nederl tijdschr v geneesk 93 (II) 19 48° (1949)

Huerga J dela and Popper H Standardized Reagent for Thymol Turbidity Test J Lab & Clin Med 34 (6) 877 880 (June) 1949

Shank R E and Hoagland C L A Modified Method for the Quantitative Determination of the Thymol Turbidity Reaction of Serum J Biol Chem 162 133 138 (Jan) 1946

Urobilinogen

Wilson T M and Davidson L S P Ehrlich s Aldehyde Test for Urobilinogen British M J 1949 884 889 (May) 1949

SECTION IX

CLINICAL LABORATORY PROCEDURES (*Cont'd*)

F FUNCTIONAL TESTS OF ENDOCRINE GASTRIC AND OTHER SYSTEMS

1 Pituitary Adrenocorticotrophic Hormone (ACTH) Test

Reference

Forsham P H, Thorn G W, Prunty F T G and Hills A G Clinical Studies With Pituitary Adrenocorticotrophin J Clin Endocrinol 8 15 66 (Jan) 1948

Principle

Following the injection of pituitary adrenocorticotrophic hormone (ACTH) in normal individuals there is an immediate and striking fall in circulating eosinophils and a rise in the uric acid excretion Patients with Addison's disease fail to show these changes

Apparatus

1 Sterile syringe and needle for intramuscular injection of ACTH
2 Apparatus for direct eosinophil count
3 Apparatus for estimating uric acid and creatinine

Reagents

1 ACTH for Intramuscular Injection The solubility varies with different batches of ACTH The hormone requires alkalinization to a pH between 8 and 9 (faint pink with phenolphthalein) To 5 ml of sterile saline add three drops of approximately N/10 NaOH This is taken up in a syringe and added to the rubber capped vial containing 25 mg of ACTH Shake gently until homogeneous A somewhat cloudy solution is obtained This should not be kept longer than twelve hours at 4 C or longer than two hours at room temperature It should be frozen for long storage The powder appears stable
2 Reagents for direct eosinophil count
3 Reagents for estimating uric acid and creatinine

Procedure

1 No food is allowed after 8 00 P M at night
2 On the following day give the patient [illegible]00 ml of water at 6 00 8 00 and 10 00 A M
3 Collect urine from 6 00 to 8 00 A M
4 An eosinophil count is done at 8 00 A M
5 Immediately after the 8 00 A M specimen inject [illegible]5 mg ACTH intramuscularly deep into the gluteus maximus A moderate degree of soreness often persists for a day or two
6 Urine is collected from 9 00 A M to 12 00 noon and the eosinophil count is repeated at noon
7 Analyze the urine specimens for uric acid and creatinine

Amyloidosis

Unger P N Zuckerbrod M Beck G J and Steele J M Study of the Disappearance of Congo Red From the Blood of Non amyloid Subjects and Patients With Amyloidosis J Clin Investigation 27 111 117 (Jan) 1948

Bile in Urine

Hawkinson V Watson C J and Turner R H Modification of Harrison s Test for Bilirubin in Urine Epecially Suited for Mass and Serial Use J A M A 129 514 515 (Oct) 1945

Cephalin Flocculation

Hanger F M Serological Differentiation of Obstructive From Hepatogenous Jaundice by Flocculation of Cephalin Cholesterol Emulsion J Clin Investigation 18 61 69 (May) 1939

Cholinesterase Activity

Vorhaus L J II and Kark R M Measurement of Serum Cholinesterase Activity A Useful Laboratory Tool in the Study of Diseases of the Liver and Biliary System To be published Med cal Nutrition Laboratory Report No 56 June 8 1949

Galactose

Colcher H Patek A J Jr and Kendall F L Galactose Di appearance From the Blood Stream Calculation of the Galacto e Removal Constant and Its Application as a Test for Liver Function J Clin Investigation 2, 768 ,,5 (Sept) 1946

Hemosiderin in Urine

Rous P Urinary Siderosis Hemosiderin Granules in the Urine as an Aid to Diagnosis of Pernicious Anemia Hemachromatosis and Other Diseases Causing Siderosis J Exper Med 28 645 658 (Nov) 1918

Hippuric Acid

Quick A J The Clinical Application of the Hippuric Acid and Prothrombin Test Am J Clin Path 10 2?2 2?3 (March) 1940

Phosphatase

Gutman A B and Hanger F M Jr Differential Diagno is of Jaundice by Combined Serum Phosphatase Determination and Cephalin Flocculation Test M Clin North America 25 83, 848 (May) 1941

Portal Pressure

Bean W B Paul W D and Franklin M Preliminary Studies on an Indirect Method for Determining Portal Pressures J Clin Investigation 28 769 ,,0 (July) 1949

Prothrombin Time

Aggeler P M Howard J Lucia S P Clark W and Astaff A Standardization of the Quick Prothrombin Test Blood 1 2 0 2?3 (March) 1946

Quick A J The Clinical S gnificance of Prothrombin as a Factor in Hemorrhage Pennsylvania M J 43 125 130 (Oct) 1939

Unger P N and Shapiro S Prothrombin Respon e to the Parenteral Administration of Large Doses of Vitamin K in Subjects With Normal Liver Function and in Cases of Liver Disease A Standardized Test for the Estimation of Hepatic Function J Clin Investigation 27 39 4, (Jan) 1948

Thymol Turbidity

Dekema, H The Thymol Turbidity Test and the Cephalin Cholesterol Flocculation Test Nederl tijdschr v geneesk 93 (II) 19,4 82 (1949)

Huerga J de la and Popper H Standardized Reagent for Thymol Turbidity Test J Lab & Clin Med 34 (6) 87 880 (June) 1949

Shank R E and Hoagland C L A Modified Method for the Quantitative Determination of the Thymol Turbidity Reaction of Serum J Biol Chem 162 133 138 (Jan) 1946

Urobilinogen

Wilson T M and Davidson L S P Ehrlich s Aldehyde Test for Urobilinogen British M J 1949 884 889 (May) 1949

Cold Pressor Tests

Markentin J and Lang J D Physicians Handbook ed 4 Palo Alto Calif 1940 University Medical Publishers page 78

Porphyria

Welcker M L The Porphyrins New England J Med 232 11 19 (Jan) 1945
Nesbitt S Acute Porphyria J A M A 124 86 94 (Jan) 1944
Watson C J Schwartz S and Hawkinson V Studies of the Uroporphyrins II Further Studies of the Porphyrins of the Urine Feces Bile and Liver in Cases of Porphyria With Particular Reference to a Waldenstrom Type Porphyrin Behaving as an Entity on the Tswett Column J Biol Chem 157 345 362 (Jan) 1945

G SUBSTANCES OF OCCASIONAL CLINICAL IMPORTANCE MISCELLANEOUS REFERENCES

Alcohol

Harger R N Raney, B B Bridwell E G and Kitchel M F The Partition Ratio of Alcohol Between Air and Water Urine and Blood Estimation and Identification of Alcohol in These Liquids From Analysis of Air Equilibrated With Them J Biol Chem 183 197 14 (March) 1950

Heparin

Astrup P On the Determination of Heparin in Blood Plasma and Urine Acta pharmacol et tox col 3 (2) 165 178 1947

Mercury

Van Suchtelen H Warmoltz N and Wiggerink G L Method for Determining the Mercury Content of Air Philips Research Repts 11 119 1949

Nicotine

Wolff William A., Hawkins Marina H and Giles W E The Spectrophotometric Estimation of Nicotine in Blood J Biol Chem 175 8 5 831 (Sept) 1948

Phenols

Schmidt E G Urinary Phenol IV The Simultaneous Determination of Phenol and p Cresol in Urine J Biol Chem 179 11 16 (May) 1949
Theis R C and Benedict S R The Determination of Phenols in the Blood J Biol Chem 61 67 72 (Aug) 1924
Volterra Mario Urinary Phenols I Method of Determination Urinary Phenols II Their Significance in Normal and Pathological Conditions Am J Clin Path 12 5 533 580 589 (Nov) 1942

Procaine

Tsing K S Coon J M and Conway A C A Spectrophotometric Method for Determination of Procaine and p Aminobenzoic Acid J Lab & Clin. Med 34 (6) 8 2 879 (June) 1949

Salicylates

Volterra Mario and Jacobs Mildred D A Simple Method for the Determination of Salicylates in Blood J Lab & Clin Med 32 (10) 1 8 1 83 (Oct) 194

Streptomycin

Marshall E K, Jr Blanchard K C and Buhle Emmett L Colorimetric Methods for Determination of Streptomycin J Pharm & Exper Therap 90 (4) 367 374 (Aug) 194

Sulfonamides

Bratton A C and Marshall E K Jr A New Coupling Component for Sulfanilamide Determination J Biol Chem 128 537 5 0 (May) 1939

Calculation

1 Calculate eosinophils uric acid and creatinine as described under those methods
2 Calculate the uric acid creatinine ratio for both specimens of urine according to the formula

$$\text{uric acid/creatinine ratio} = \frac{\text{mg uric acid/100 ml urine}}{\text{mg creatinine/100 ml urine}}$$

3 Calculate the percentage change of eosinophils and uric acid/creatinine ratio between the pre injection and post injection values according to the formula

$$\%\ \text{change} = \frac{(\text{Pre injection value} - \text{post injection value}) \times 100}{(\text{Pre injection value})}$$

Interpretation

Eosinophil Count Normal subjects have a decrease of 70% or more 50% is the lower limit of normal Patients with Addison s disease show little or no drop in eosinophil count

Uric Acid Creatinine Ratio Normal subjects will increase their uric acid creatinine ratio by approximately 100 percent average Patients with Addison s disease show approximately a 20 percent increase An increase of over 50 percent is strong evidence against adrenal insufficiency

2 References to Endocrine Gastric and Other Functional Tests

Water and Salt Tests of Adrenal Sufficiency

Robinson, F J Power M H and Kepler E J Two New Procedures to Assist in the Recognition and Exclusion of Addison s Disease Proc Staff Meet, Mayo Clin 16 577 583 (Sept) 1947

Cutler H H Power M H and Wilder R M Concentration of Chloride Sodium and Potassium in Urine and Blood Their Diagnostic Significance in Adrenal Insufficiency J A M A 111 117 122 (Jan) 1938

Adrenal Hormones

Fraser R W Forbes A P Albright F Sulkowitch S and Reifenstein E C Jr Colorimetric Assay of 17 Ketosteroids in Urine J Clin Endocrinol 1 234 255 (March) 1941

Talbot N B Saltzman F Wixon R L and Wolfe J K Colorimetric Assay of Urinary Corticosteroid like Substances J Biol Chem 160 535 546 (Oct) 1945

Pancreas

Fraser R Albright F and Smith P H The Value of Glucose Tolerance Tests Insulin Tolerance Test and the Glucose Insulin Tolerance Test in the Diagnosis of Endocrinological Disorders of Glucose Metabolism J Clin Endocrinol 1 297 306 (April) 1941

Comfort M W and Osterberg A E Serum Amylase and Serum Lipase in the Diagnosis of Diseases of the Pancreas M Clin North America 24 1137 1149 (July) 1940

Beazell J M Schmidt C R and Ivy A C The Diagnosis and Treatment of Achylia Pancreatica J A M A 116 736 739 (June) 1941

Parathyroids

Albright F The Parathyroids—Physiology and Therapeutics J A M A 117 527 533 (Aug) 1941

Gastric Function

Glenn P M Histamine Stimulated Fractional Gastric Analysis The Diagnostic Value of Total Secretion Gastroenterology 6 409 416 (May) 1946

Hollander F The Insulin Test for the Presence of Intact Nerve Fibers After Vagal Operations for Peptic Ulcer Gastroenterology 7 607 614 (Dec) 1946

Vanzant F R and Alvarez W C Calculating the Diagnostic Value of Gastric Analysis A Study in the Methodology of Diagnosis Am J Digest Dis & Nutrition 2 466 47- (Oct) 1935

Yule C Udny An Introduction to the Theory of Statistics London England 1940 Charles Griffin and Company Ltd
This text covers the theory of statistics has an excellent bibliography and gives answers to problems presented in the text

2 General Handbooks

Handbook of Chemistry and Physics Cleveland Ohio 1947 Chemical Rubber Publishing Company

Handbook of Chemistry edited by Lange Adolph N Sandusky Ohio 1946 Handbook Publishers Inc

Van Nostrand s Chemical Annual edited by Olsen John C New York 1934 D Van Nostrand Company Inc

Marks Lionel Mechanical Engineer s Handbook New York 1941 McGraw Hill Book Co Inc

SECTION X

STATISTICAL METHODS

A REFERENCES TO STANDARD WORKS

1 Textbooks of Statistics

Buros O car Krisen Research and Stati tical Methodology New Brun wick N J 19 8 Rutgers University Press
An annotated bibliography

Buros Oscar Krisen The Second Yearbook of Research and Statistical Methodology—Books and Reviews Highland Park N J 1941 The Gryphon Press
An annotated bibliography

Cramer H Math matical Method of Statistics Princeton N J 1946 Princeton University Press
Very theoretical

Croxton F E and Cowden D J Applied General Statistics New York 1946 Prentice Hall Inc
Gives a good pre entation of statistical methods and their application in various fields

Ezekiel Mordecai Methods of Correlation Analysis ew York 1941 John Wiley & Sons Inc
Covers that part of the field of stati tics wh ch is concerned with tudying the relations between variables

Fisher Ronald A Statistical Methods for Re earch Workers Edinburgh Scotland 194- Oliver and Boyd, Ltd. pp xiii 339
A comprehensive text with emphasis on biology

Fisher Ronald A and Yates Frank Statistical Tables for Biological Agricultural and Medical Research, Edinburgh Scotland 1938 Oliver & Boyd Ltd pp 90

Goodwin H M Elements of the Preci ion of Measurements and Graphical Method McGraw Hill Book Co N Y 1919
An excellent account of the subject of making measurements of errors involved, and of the statistical methods of handling instrumental data There is also a section dealing with graphical methods Illustrative problems are given together with examples

Kenney John F Mathematics of Statistics New York 1939 D Van Nostrand Company Inc
An excellent dictionary

Kurtz Albert K and Edgeston Harold A Stati tical Dictionary of Terms and Symbols New York 1939 John Wiley & Sons Inc pp xiii 191
An excellent dictionary

Mainland Donald The Treatment of Clinical and Laboratory Data An Introduction to Statistical Ideas and Method for Medical and Dental Workers 19-8 Edinburgh Scotland Oliver & Boyd Ltd pp xi 40
This book has been prepared for the express purpose of aiding the clinician untrained in statistics in the evaluation of the significance of laboratory observations It is an appeal to understanding rather than to mathemat cal derivation

Rider, P R An Introduction to Modern Statistical Methods New York, 1939, John Wiley and Sons Inc
A practical statistical book that has proved u eful in handling biological data.

Snedecor George W Stati tical Methods Applied to Experiments in Agriculture and Biology ed 3 Ames Iowa, 1940, Iowa State College Pre s pp xv 4--
A tandard textbook

B CONVERSION BETWEEN DIFFERENT SYSTEMS OF WEIGHTS AND MEASURES

1 Length—Metric System

Angstrom Units Å	Millimicrons mμ	Microns μ	Millimeters mm	Centimeters cm
1	0.1	10	10	10^{-8}
10	1	10^{-3}	10^{-6}	10^{-7}
10	10^{3}	1	10^{-3}	10

Millimeters mm	Centimeter cm	Meter m	Kilometers km	U.S. Equivalents
1	0.1	0.001	10^{-6}	0.0393 inch
10	1	0.01	10^{-5}	0.393 inch
1000	100	1	10^{-3}	39.37 inch 3.2808 feet
10	10	1000	1	0.6137 mile

2. Length—U.S. System

Inches in	Feet ft	Yards yd	Miles mi	Metric Equivalent
1	0.08333	0.02778		2.5401 cm
12	1	0.3333		0.30480 m
36	3	1	0.000568	0.91440 m
63 360	5280	1760	1	1.60935 km

3 Area—Metric System

Square Millimeters mm²	Square Centimeters cm²	Square Meter m²	Square Kilometers km²	U.S. Equivalents
1	0.01	10	10^{-12}	0.00155 sq in.
100	1	10	10^{-10}	0.15500 sq in
10	10 000	1	10	10.638 sq ft
10	10^{10}	10^{6}	1	

4 Area—U.S. System

Square Inches sq in	Square Feet sq ft	Square Yards sq yd	Acres A	Square Miles sq mi	Metric Equivalent
1	0.006944		–	–	6.4516 cm²
144	1	0.1111	–	–	0.0929 m²
1296	9	1	–	–	0.8361 m²
	43,560	4840	1	0.001563	4046.97 m²
		3097 600	640	1	2.5898 km²

5 Volume—Metric System

Cubic Millimeters mm³	Cubic Centimeters cm³	Cubic Meters m³	U.S. Equivalent
1	0.001	10	0.000061 cu in
1000	1	10^{-6}	0.0610 cu in
10^{9}	10	1	35.3144 cu ft 1.3094 cu yd

6 Volume—U.S. System

Cubic Inches cu in	Cubic Feet cu ft	Cubic Yards cu yd	Metric Equivalent
1	0.000578		16.387 cm³
1728	1	0.03704	0.028317 m³
46 656	27	1	0.7646 m³

SECTION XI

MISCELLANEOUS DATA

A ATOMIC WEIGHTS

References

Table of International Atomic Weights 1946

1947 Summary of Nuclear Data (Aug 1946–Nov 1947) Compiled by Nuclear Data Committee of Clinton National Laboratory Nucleonics 2 Part 2 (May) 1948

TABLE 2 — ATOMIC WEIGHTS

ELEMENT	SYMBOL	ATOMIC NO	ATOMIC WT	ELEMENT	SYMBOL	ATOMIC NO	ATOMIC WT
Actinium	Ac	89	227 ca	Neodymium	Nd	60	144.27
Alabamine	Ab	85	1 ca	Neon	Ne	10	20.183
Aluminum	Al	13	26.97	Neptunium	Np	93	239 ca.
Americium	Am	95	241 ca	Nickel	Ni	28	58.69
Antimony	Sb	51	121.76	Nitrogen	N		14.008
Argon	A	18	39.944	Osmium	Os	76	190
Arsenic	As	33	74.91	Oxygen	O	8	*16.0000*
Astatine	At	85	216 ca	Palladium	Pd	46	106
Barium	Ba	56	137.36	Phosphorus	P	15	30.98
Beryllium	Be	4	9.0	Platinum	Pt	78	195.23
Bismuth	Bi	83	209.00	Plutonium	Pu	94	239 ca
Boron	B	5	10.8	Polonium	Po	84	210 ca
Bromine	Br	35	79.916	Potassium	K	19	39.096
Cadmium	Cd	48	112.41	Praseodymium	Pr	59	140.92
Calcium	Ca	20	40.08	Protoactinium	Pa	91	231
Carbon	C	6	12.01	Radium	Ra	88	226.05
Cerium	Ce	58	140.13	Radon	Rn	86	222
Cesium	Cs	55	132.91	Rhenium	Re	75	186.31
Chlorine	Cl	17	35.457	Rhodium	Rh	45	102.91
Chromium	Cr	24	52.01	Rubidium	Rb	37	85.48
Cobalt	Co	27	58.94	Ruthenium	Ru	44	101
Columbium	Cb	41	92.91	Samarium	Sm	62	150.43
Copper	Cu	29	63.57	Scandium	Sc	21	45.10
Curium	Cm	96	241 ca.	Selenium	Se	34	78.96
Dysprosium	Dy	66	162.46	Silicon	Si	14	28.06
Erbium	Er	68	167.2	Silver	Ag	47	107.880
Europium	Eu	63	152.0	Sodium	Na	11	22.997
Fluorine	F	9	19.000	Strontium	Sr	38	87.63
Francium	Fr	87	223 ca	Sulfur	S	16	32.06
Gadolinium	Gd	64	156.9	Tantalum	Ta	73	180.88
Gallium	Ga	31	69.72	Technetium (Masurium)	Tc	43	96 ca
Germanium	Ge	32	72.60	Tellurium	Te	52	127.61
Gold	Au	79	197.2	Terbium	Tb	65	159
Hafnium	Hf	72	178.6	Thallium	Tl	81	204.39
Helium	He	2	4.003	Thorium	Th	90	232.12
Holmium	Ho	67	164.94	Thulium	Tm	69	169.4
Hydrogen	H	1	1.0080	Tin	Sn	50	118
Illinium	Il	61	146 ca	Titanium	Ti	22	47.90
Indium	In	49	114.76	Tungsten	W	74	183.92
Iodine	I	53	126.92	Uranium	U	92	238.07
Iridium	Ir	77	193.1	Vanadium	V	23	50.95
Iron	Fe	26	55.85	Virginium	Vi	87	224 ca.
Krypton	Kr	36	83.7	Xenon	Xe	54	131.3
Lanthanum	La	57	138.92	Ytterbium	Yb	70	173.04
Lead	Pb	82	207.21	Yttrium	Y	39	88.92
Lithium	Li	3	6.940	Zinc	Zn	30	65.38
Lutecium	Lu	71	174.99	Zirconium	Zr	40	91.22
Magnesium	Mg	12	24.32	Element 61	—	61	148 ca
Manganese	Mn	25	54.93	Element 86	—	86	—
Mercury	Hg	80	200.61				
Molybdenum	Mo	42	95.95				

B CONVERSION BETWEEN DIFFERENT SYSTEMS OF WEIGHTS AND MEASURES

1 Length—Metric System

Angstrom Units Å	Millimicrons mμ	Microns μ	Millimeters mm	Centimeters cm
1	0.1	10	10^{-7}	10^{-8}
10	1	10^{-3}	10^{-6}	10
10^4	10^3	1	10^{-3}	10
Millimeters mm	**Centimeter cm**	**Meter m**	**Kilometers km**	**U. S. Equivalents**
1	0.1	0.001	10^{-6}	0.03937 inch
10	1	0.01	10^{-5}	0.393 inch
1000	100	1	10^{-3}	39.3 inch; 3.2808 feet
10	10	1000	1	0.62137 mile

2. Length—U. S. System

Inches in	Feet ft	Yards yd	Miles mi	Metric Equivalent
1	0.08333	0.0278		2.54001 cm
1	1	0.33333		0.304801 m
36	3	1	0.000568	0.91440 m
63 360	5280	1760	1	1.6093 km

3 Area—Metric System

Square Millimeters mm	Square Centimeters cm²	Square Meters m	Square Kilometers km²	U. S. Equivalents
1	0.01	10^{-6}	10^{-12}	0.00155 sq in
100	1	10^{-4}	10	0.1550 sq in
10^6	10 000	1	10^{-6}	10.638 sq ft.
10^{12}	10^{10}	10^6	1	

4 Area—U. S. System

Square Inches sq in	Square Feet sq ft	Square Yards sq yd	Acres A	Square Miles sq mi	Metric Equivalent
1	0.006944		–	–	6.4516 cm²
144	1	0.1111	–	–	0.0929 m²
1296	9	1	–	–	0.8361 m²
	43,560	4840	1	0.001563	4048.93 m²
		309 600	640	1	2.5898 km²

5 Volume—Metric System

Cubic Millimeters mm³	Cubic Centimeters cm³	Cubic Meters m³	U. S. Equivalent
1	0.001	10^{-9}	0.000061 cu in
1000	1	10^{-6}	0.0610 cu in
10^9	10	1	35.3144 cu ft; 1.3094 cu yd

6 Volume—U. S. System

Cubic Inches cu in	Cubic Feet cu ft	Cubic Yards cu yd	Metric Equivalent
1	0.000578		16.38 cm³
1728	1	0.03703	0.028317 m³
46 656	27	1	0.7646 m³

7 Capacity—Metric System

Milliliters ml	Liters l	U S Equivalent
1	0 001	16 31 minims 0 0610 cu in
1000	1	0 318 fl dram 33 8147 fl ounce

8 Capacity—U S System—Liquid

Gills gi	Pints pt	Quarts qt	Gallons	Metric Equivalent
1	0 5	0 1 5	0 031 5	118 .9? ml
4	1	0 5	0 1.5	0 4.3? l
8	.	1	0 5	0 9463 l
3.	8	4	1	3 .8.3 l

9 Capacity—U S System—Dry

Pints pt	Quarts qt	Peck pk	Bushels bu	Metric Equivalent
1	0 5	0 06 5	0 0156.5	0 5506 l
.	1	0 1.5	0 031?5	1 101? l
16	8	1	0 25	8 8096 l
64	3	4	1	35.2383 l

10 Mass—Metric System

Milligrams mg	Grams gm	Kilogram kg	U S Equivalent
1	0 001	10^{-6}	0 01543 grain
1000	1	0 001	15 43 36 grain
10^6	1000	1	0 035274 oz av 2 2046 lb av

11 Mass—U S System—Avoirdupois Commercial

Grain gr	Drams dr av	Ounces oz av	Pounds lb av	Ton short tn	Metric Equivalent
1	0 0365	–	–	–	0 0648 gm
..4375	1	0 06?	–	–	1 .. 13 gm
43.	16	1	0 06 5	–	8 349. gm
000	? 6	16	1	0 0005	0 4.36 kg
.	–	? 000	000	1	90 18 kg

12 U S Apothecaries Fluid Measure

Minim min	Fluid Drams fl dr	Fluid Ounces fl oz	Pints pt	Metric Equivalent
1	0 01666	0 00 08	–	0 0616 ml
60	1	0 1?5	–	3 69 ml
480	8	1	0 06 5	.9 573 ml
.680	1?8	16	1	3 .8.3 l

13 U S Apothecaries Weight

Grain gr	Scruples s ap	Drams dr ap	Ounces oz ap	Pounds lb ap	Metric Equivalent
1	0 05	0 0166	–	–	64 9? mg
?0	1	0 333	0 04167	–	1 9. gm
60	.	1	0 1 5	0 0104	3 888 gm
480	.4	8	1	0 08333	31 103 gm
. 60	?88	96	1	1	3 3?4 gm

14 Time

Seconds sec	Minutes min	Hours hr	Days da.	Years yr
2	0 01667	0 000 278		–
60	1	0 166		–
3 600	60	1	0 041667	
86 400	1440	4	1	–
–			365 4	1 common
–			36. 56	1 sidereal

7 Capacity—Metric System

Milliliters ml	Liters l	U S Equivalent
1	0 001	16 31 minims 0 0610 cu
1000	1	0 518 fl dram 33 8147 fl ounces

8 Capacity—U S System—Liquid

Gill gi	Pint pt	Quarts qt	Gallons	Metric Equivalent
1	0 5	0 1 5	0 031 5	118 9 ml
4	1	0 5	0 1 5	0 4 3 l
8		1	0 5	0 9463 l
32	8	4	1	85 l

9 Capacity—U S System—Dry

Pints pt	Quart qt	Pecks pk	Bushels bu	Metric Equivalent
1	0	0 06 5	0 0156 5	0 5506 l
	1	0 1 5	0 031 5	1 1012 l
16	8	1	0 25	8 8096 l
64	3	4	1	35 383 l

10 Mass—Metric System

Milligrams mg	Grams gm	Kilograms kg	U S Equivalent
1	0 001	10-6	01543 grain
1000	1	0 001	15 4336 grain
10	1000	1	0 035 74 oz av 046 lb a

11 Mass—U S System—Avoirdupois Commercial

Grains gr	Drams dr av	Ounces o a	Pound lb a	Ton short tn	Metric Equivalent
1	03 5				0 0648 gm
2 34 5	1	0 062			1 7 18 gm
43	16	1	0 06		8 3495 gm
000	6	16	1	0 0005	0 4 36 kg
		32000	2000	1	90 18 kg

12 U S Apothecaries Fluid Measure

Minims min	Fluid Drams fl dr	Fluid Ounces fl oz	Pints pt	Metric Equivalent
1	1666	00 08		0 0616 ml
60	1	0 125		3 697 ml
480	8	1	0 06 5	25 3 ml
680	1 8	16	1	3 853 l

13 U S Apothecaries Weight

Grain gr	Scruples s ap	Drams dr ap	Ounces oz ap	Pounds lb ap	Metric Equivalent
1	0 05	0 0166			64 9 mg
0	1	0 333	0 0416		1 96 gm
60	3	1	0 12	0 0104	3 888 g
480	24	8	1	0 08333	31 103 gm
5 60	288	96	1	1	3 3 4 gm

Time

Seconds sec	Minutes min	Hours hr	Days da	Years yr
1	0.01667	0.000[illegible]78	–	–
60	1	0.0166	–	–
3 600	60	1	0.04166	–
86 400	1440	[illegible]4	1	–
–	–	–	36[illegible]	1 common
–	–	–	36[illegible]6	1 sidereal

7 Capacity—Metric System

Milliliters ml	Liters l	U S Equivalent
1	0 001	16 [illegible]31 minims 0 0[illegible]10 cu in
1000	1	[illegible]0 [illegible]18 fl dram 33 8147 fl ounces

8. Capacity—U S System—Liquid

Gill gi	Pints pt	Quart qt	Gallons	Metric Equivalent
1	0 [illegible]	0 1 [illegible]	0 031[illegible]	118 [illegible] ml
4	1	0 [illegible]	0 1[illegible]	0 4 3 l
8		1	0 [illegible]	0 9463 l
32	8	4	1	3 [illegible]3 l

9 Capacity—U S System—Dry

Pints pt	Quarts qt	Pecks pk	Bushels bu	Metric Equivalent
1	0 [illegible]	0 06 [illegible]	0 01[illegible]	0 [illegible]06 l
	1	0 1 [illegible]	0 031[illegible]	1 101[illegible] l
16	8	1	0 [illegible]5	8 8096 l
64	3	4	1	3[illegible] [illegible] l

10 Mass—Metric System

Milligrams mg	Grams gm	Kilograms kg	U S Equivalents
1	0 001	10	0 01543 grain
1000	1	0 001	1[illegible] 43 [illegible] grain
10^6	1000	1	0 03[illegible]274 oz av 2 [illegible]046 l av

11 Mass—U S System—Avoirdupois Commercial

Grain gr	Dram dr av	Ounces oz av	Pound lb av	Ton short tn	Metric Equivalent
1	0 036		–	–	0 0648 gm
[illegible] 43 [illegible]	1	0 06		–	1 [illegible] gm
43	16	1	0 06 [illegible]	–	28 [illegible] gm
[illegible]000	[illegible]6	16	1	0 000[illegible]	0 4[illegible]36 kg
–		[illegible]000	[illegible]000	1	90 [illegible] kg

12 U S Apothecaries Fluid Measure

Minim min	Fluid Drams fl dr	Fluid Ounces fl oz	Pints pt	Metric Equivalent
1	*0 016[illegible]*	*0 00[illegible]08*	–	0 06[illegible] ml
60	1	0 1[illegible]	–	3 69 ml
480	8	1	0 06 [illegible]	[illegible]9 5[illegible]3 ml
[illegible]	1 8	16	1	3 [illegible]3 l

13 U S Apothecaries Weight

Grain gr	Scruple s ap	Dram dr ap	Ounces oz ap	Pounds lb ap	Metric Equivalent
1	0 0[illegible]	0 0166	–	–	64 99 mg
[illegible]0	1	0 3[illegible]	0 041[illegible]7	–	1 [illegible]96 gm
[illegible]0	3	1	0 125	0 0104	3 [illegible] gm
480	24	8	1	0 08333	31 103 gm
[illegible] 60	[illegible]88	96	1[illegible]	1	373 4 gm

14 Time

Seconds sec	Minutes min	Hours hr	Days da	Years yr
1	0 01667	0 000 778	–	–
60	1	0 0166	–	–
3 600	60	1	0 04166	–
86 400	1440	24	1	–
–	–	–	365 242	1 common
–	–	–	365 256	1 sidereal

C ELEMENTS LISTED IN ORDER OF ATOMIC NUMBER

TABLE 28

ELEMENTS LISTED IN ORDER OF ATOMIC NUMBER

(For mass numbers and % abundance of stable isotopes see International Tables of Stable Isotopes. For mass number and half life of unstable isotopes see 1947 Summary of Nuclear Data Nucleonics 2 Part 2 May 1948.)

ATOMIC NO	NAME	SYMBOL	ATOMIC NO	NAME	SYMBOL
0	Neutron	n	48	Cadmium	Cd
1	Deuterium	D	49	Indium	In
1	Hydrogen	H	50	Tin	Sn
2	Helium	He	51	Antimony	Sb
3	Lithium	Li	52	Tellurium	Te
4	Beryllium	Be	53	Iodine	I
5	Boron	B	54	Xenon	Xe
6	Carbon	C	55	Cesium	Cs
7	Nitrogen	N	56	Barium	Ba
8	Oxygen	O	57	Lanthanum	La
9	Fluorine	F	58	Cerium	Ce
10	Neon	Ne	59	Praseodymium	Pr
11	Sodium	Na	60	Neodymium	Nd
12	Magnesium	Mg	61	Element 61	—
13	Aluminum	Al	62	Samarium	Sm
14	Silicon	Si	63	Europium	Eu
15	Phosphorus	P	64	Gadolinium	Gd
16	Sulfur	S	65	Terbium	Tb
17	Chlorine	Cl	66	Dysprosium	Dy
18	Argon	A	67	Holmium	Ho
19	Potassium	K	68	Erbium	Er
20	Calcium	Ca	69	Thulium	Tm
21	Scandium	Sc	70	Ytterbium	Yb
22	Titanium	Ti	71	Lutecium	Lu
23	Vanadium	V	72	Hafnium	Hf
24	Chromium	Cr	73	Tantalum	Ta
25	Manganese	Mn	74	Tungsten	W
26	Iron	Fe	75	*Rhenium*	*Re*
27	Cobalt	Co	76	Osmium	Os
28	Nickel	Ni	77	Iridium	Ir
29	Copper	Cu	78	Platinum	Pt
30	Zinc	Zn	79	Gold	Au
31	Gallium	Ga	80	Mercury	Hg
32	Germanium	Ge	81	Thallium	Tl
33	Arsenic	As	82	Lead	Pb
34	Selenium	Se	83	Bismuth	Bi
35	Bromine	Br	84	Polonium	Po
36	Krypton	Kr	85	Astatine	At
37	Rubidium	Rb	86	—	—
38	Strontium	Sr	87	Francium	Fr
39	Yttrium	Y	88	Radium	Ra
40	Zirconium	Zr	89	Actinium	Ac
41	Columbium	Cb	90	Thorium	Th
42	Molybdenum	Mo	91	Protoactinium	Pa
43	Technetium	Tc	92	Uranium	U
44	Ruthenium	Ru	93	Neptunium	Np
45	Rhodium	Rh	94	Plutonium	Pu
46	Palladium	Pd	95	Americium	Am
47	Silver	Ag	96	Curium	Cm

D COMPOSITION OF BODY FLUIDS

In metabolic studies it is sometimes helpful to find in a convenient form and in one place average values for various components of the body The four accompanying tables (Tables 29 30 31 and 32) list for whole blood plasma urine and feces various constituents in several general categories These categories are (1) water (2) hydrogen ion concentration (3) minerals (4) nitrogen and protein derivatives (5) carbohydrates and derivatives (6) fats and derivatives (7) vitamins (8) hormones (9) enzymes (10) pigments (11) gases and (12) measurements of clinical interest

It is hardly necessary to warn the reader that the term normal range is notoriously difficult to define that the concentration of any substance may vary from person to person that the effects of many factors such as age sex disease previous diet exercise and season may be large and that no table can hope to be complete

TABLE 29 COMPOSITION OF WHOLE BLOOD

(Listed below are normal ranges for various substances in whole blood all values are for healthy individuals in a resting condition at sea level in a temperate environment The values for many substances are closely related to dietary intake and to exercise)

SUBSTANCE	UNITS	USUAL RANGE
1 *Water*		
Specific gravity	vs water	1 035 1 075
Total solids	gm/100 ml	18 22
Water	ml/100 ml	78 82
2 *Hydrogen Ion Concentration*		
Hydrogen ion concentration arterial	pH	7 35 7 45
Hydrogen ion concentration venous	pH	7 25 7 35
3 *Minerals*		
Aluminum	mg/100 ml	0 1 0 2
Bromine	mg/100 ml	0 2 0 3
Chloride	mEq/liter	83 86
Iodine	*mcg/100 ml*	*0 6 8 4*
Magnesium	mg/100 ml	1 0 2 0
Manganese	mcg/100 ml	0 1 0 3
Rubidium	mg/100 ml	0 2 0 4
Silicon	mg SiO_2/100 ml	0 1
Zinc	mg/100 ml	0 6 1 4
4 *Nitrogen and Protein Derivatives*		
Alanine	mg/100 ml	2 8 5 0
Ergothioneine	mg/100 ml	3 9 17
Glycine	mg/100 ml	1 8 2 3
Nonprotein nitrogen	mg/100 ml	30 40
Urea nitrogen	mg/100 ml	10 15
Uric acid	mg/100 ml	2 4
5 *Carbohydrates and Derivatives*		
Acetaldehyde	mg/100 ml	0 2 0 6
Alpha ketoglutaric acid	mg/100 ml	0 1 0 3
Citric acid	mg/100 ml	1 3 2.3
Glucose	mg/100 ml	70 110
Glycogen	mg/100 ml	1 16
Lactic acid	mg/100 ml	5 20
Oxalic acid	mg/100 ml	0 9 1 5
Pyruvic acid	mg/100 ml	0 1
6 *Fats and Derivatives*		
Acetoacetic acid	mg/100 ml	0 1 0
Acetone	mg/100 ml	0-0 1
Beta hydroxybutyric acid	mg/100 ml	0 5 1 0
Cholic acid	*mg/100 ml*	*0 2 3 0*
7 *Vitamins*		
Ascorbic acid	mg/100 ml	0 5 1 5
Biotin	mcg/100 ml	1 2
Cocarboxylase	mcg/100 ml	5 10
Folic acid	mcg/100 ml	0 5 1 3
Niacin	mg/100 ml	0 6 0 8
Pantothenic acid	mcg/100 ml	1 47
Riboflavin	mcg/100 ml	20 60
Thiamine	mcg/100 ml	3 9
Tocopherols	mg/100 ml	0.5 1 8
Tryptophane	mg/100 ml	250 300
Vitamin B	mμg/ml	0 6 1 4
8 *Hormones*		
Adrenaline	micro mcg/ml	20 80
Histamine	mcg/100 ml	1 10

TABLE 9—CONT'D

9 *Enzymes*		
Amylase	units/ml	30 90
Arginase	units/ml	8 16
Carbonic anhydrase	Roughton units/ml	1 ? 6
Phosphatase (acid)	Bodansky units	10 ?0
Zin anhydrase	mcg/ml	? 10
10 *Pigments*		
CO Hemoglobin	gm/100 ml	0 1 0 ?
Hemoglobin (men)	gm/100 ml	14 18
Hemoglobin (women)	gm/100 ml	1 16
Methemoglobin	gm/100 ml	0 03 0 13
Sulfhemoglobin	gm/100 ml	0 1 0 3
11 *Gases*		
Arterial saturation	per cent	93 98
Carbon dioxide (arterial)	ml/100 ml STP	45 55
CO capacity (T_{∞})	mEq/liter	19 5 ?3
Carbon monoxide (arterial)	ml/100 ml STP	0 1 0 5
Nitrogen (arterial)	ml/100 ml STP	1
Oxygen (arterial)	ml/100 ml STP	18 ?
pCO	mm Hg	40-4
12 *Measurements of Clinical Interest*		
Hematocrit (men)	ml/100 ml	4 5?
Hematocrit (women)	ml/100 ml	? 46
Mean corpuscular hemoglobin concentration	per cent	33 38
Mean corpuscular volume	cu microns	?0 94
Phenols	mg/100 ml	0 7 1 8
Phenols (free)	mg/100 ml	0 05 0 09
Sedimentation rate	ml/min	0 1 0 3
Urethane	mg/100 ml	0 1 1 4

TABLE 30 COMPOSITION OF PLASMA (OR SERUM)

(Listed below are normal ranges for various substances in human plasma all values are for healthy individuals in a resting condition at sea level in a temperate environment The values for many substances are closely related to dietary intake and to exercise)

SUBSTANCE	UNITS	USUAL RANGE
1 *Water*		
Specific gravity	vs water	1 015 1 035
Total solids	gm/100 ml	8 10
Water	ml/100 ml	90 9
2 *Hydrogen Ion Concentration*		
Hydrogen ion concentration arterial	pH	[illegible] 7 45
Hydrogen ion concentration venous	pH	[illegible] 7 35
3 *Minerals*		
Aluminum	mg/100 ml	0 05 0 1
Arsenic	mcg/100 ml	0 [illegible]0
Bromine	mg/100 ml	0 2 0 6
Calcium	mg/100 ml	9 0 11 [illegible]
Chloride	mEq/liter	9[illegible] 110
Cobalt	mcg/100 ml	[illegible] 10
Copper	mcg/100 ml	[illegible] [illegible]50
Iodine (protein bound)	mcg/100 ml	4 8
Iron	mcg/100 ml	100 150
Lead	mg/100 ml	0 0 1
Magnesium	mg/100 ml	1 3
Manganese	mcg/100 ml	0 1 0 3
Phosphorus (as inorganic P)	mg/100 ml	3 4
Potassium	mEq/liter	4 6 5 8
Sodium	mEq/liter	138 143
Sulfate (inorganic)	mg/100 ml	0 9 1 1
Thiocyanate	mg/100 ml	0 [illegible] 0 8
Total base	mEq/liter	155 165
Total sulphur	mg/100 ml	90 1 0
Zinc	mcg/100 ml	1[illegible]7 900
4 *Nitrogen and Protein Derivatives*		
Albumin	gm/100 ml	4 5 [illegible]5
Alpha amino nitrogen	mg/100 ml	3 2 [illegible]5
Alpha globulin	gm/100 ml	0 7 1 5
Amino acid nitrogen	mg/100 ml	5 8
Arginine	mg/100 ml	5
Beta globulin	gm/100 ml	0 65 1 3
Creatine	mg/100 ml	3 [illegible]
Creatinine	mg/100 ml	1 2
Cystine	mg/100 ml	80 400
Fibrinogen	gm/100 ml	0 2 0 4
Free amino acid nitrogen	mg/100 ml	3
Gamma globulin	gm/100 ml	1 0 1 4
Globulin (total)	gm/100 ml	1 5 3 5
Glutamic acid	mg/100 ml	0 4 1 7
Glutamine	mg/100 ml	6 1
Glutathione	mg/100 ml	40 68
Histidine	mg/100 ml	1 1 [illegible] 1
Isoleucine	mg/100 ml	1 [illegible] 2 3
Leucine	mg/100 ml	1 6 [illegible] 6
Lysine	mg/100 ml	1 3 3 [illegible]
Methionine	mg/100 ml	100 120
Nonprotein nitrogen	mg/100 ml	[illegible] 3[illegible]
Protein total	gm/100 ml	6 [illegible] 7 [illegible]
Phenylalanine	mg/100 ml	0 01 0 03
Sulfhydryl groups	mg cysteine/100 ml	6 7
Threonine	mg/100 ml	1 3 3
Tyrosine	mg/100 ml	1 0 [illegible]
Tryptophane	mg/100 ml	1
Urea nitrogen	mg/100 ml	10 [illegible]8
Uric acid	mg/100 ml	3 6
Valine	mg/100 ml	3-4

TABLE 30—CONT'D

5 *Carbohyd ates and Derivatives*		
Acetaldehyde	mg/100 ml	0 0 0 04
Alpha ketoglutaric acid	mg/100 ml	0 1-0 3
Citric acid	mg/100 ml	1 6 [illegible]
Lactic acid	mg/100 ml	[illegible] 0
Oxalic acid	mg/100 ml	1 0 0
Pyruvic acid	mg/100 ml	0 [illegible] 0
6 *Fats and Derivatives*		
Acetoacetic acid	mg/100 ml	0 1 0 3
Acetone	mg/100 ml	0 0 1
Beta hydroxybutyri acid	mg/100 ml	0.[illegible] 1 5
Cholesterol esters	mg/100 ml	10[illegible] 155
Cholesterol (free)	mg/100 ml	3 100
Cholesterol (total)	mg/100 ml	130 330
Esterified fatty ac ds	mEq/liter	13
Ethanolamine	*mg/100 ml*	*40 50*
Lecithin	mg/100 ml	8 10
Linolenic acid	mg/100 ml	0
Lipid chlorine	*mEq/liter*	*7 5 7*
Phospholipids	mg P/100 ml	1 14
Total acetone bodies	mg/100 ml	0 8 5 0
Total lipids	mg/100 ml	[illegible]00 600
7 *Vitamins*		
Ascorbi acid	mg/100 ml	0 [illegible] 1 5
Beta-carotene	mcg/100 ml	100 [illegible]0
Biotin	mcg/100 ml	0 9[illegible] 1 66
Choline	mg/100 ml	0 5 1 5
Desoxyribonuclease	*units*	*0 14 0 54*
Flavine adenine dinucleotide	m g/100 ml	1 5 3 0
Folic ac d	m g/100 ml	1 6 1 9
Free B monophosphate (FMN)	*m g/100 ml*	*0 3 1 3*
Inositol	mg/100 ml	0 4 0 8
Niacin	mg/100 ml	0 6 0 78
Pantothenic a d	mcg/100 ml	6
Riboflavin	mcg/100 ml	1 9
Thiamine	mcg/100 ml	1 0 3 [illegible]
Tocopherols	mg/100 ml	0 [illegible] 1 8
Tryptophane	mg/100 ml	106 1 [illegible]
Vitamin A	mcg/100 ml	[illegible] 60
8 *Hormones*		
Adrenal ne	r m g/100 ml	[illegible] 10
9 *Enzymes*		
Amylase	unit /100 ml	15 3[illegible]
Beta glucuronidase	units/100 ml	0 150
Catalase	peroxide units	4 10
Choline tera e	Michel units	0 7 1 4
Diasta e	unit /100 ml	9 64
Lipase	ml of N/ 0 NaOH	bel 1 ml
Peptidase	units/ml	6 8
Phosphatase a id	Bodansky un t	[illegible] or 1 [illegible]
Pho phata alkaline	Bodansky units	[illegible] or less
Tributyrina e	Goldste n units	90 150
10 *Pigments*		
Bil ub n (direct)	mg/100 ml	0 0 4
Bil rubin (total)	mg/100 ml	0 4 0 9
11 *Fixed Ca es*		
Carbon d oxi le	mEq/liter	[illegible] 30
1 *Measu ments of Clinical Int st*		
Phenols (conjugated)	mg/100 ml	0 1 0 3
Phenols (free)	mg/100 ml	0 04 0 1
Prothrombin (dilution method)	seconds	3[illegible]-45
Prothrombin (Quick s	seconds	1 1

TABLE 31 COMPOSITION OF URINE

(Listed below are normal ranges for various substances in human urine all values are for healthy individuals in a resting condition at sea level in a temperate environment The values for many substances are closely related to dietary intake and to exercise)

SUBSTANCE	UNITS	USUAL RANGE
1 *Water*		
Specific gravity	vs water	1 008 1 030
Total solids	gm/100 ml	1 7
Water	ml/100 ml	95 99
2 *Hydrogen Ion Concentration*		
Hydrogen ion concentration	pH	4 8 7.5
3 *Minerals*		
Bromine	mg/day	1 5
Calcium	gm/day	0 2 0 5
Chloride (as NaCl)	gm/day	10 15
Chromium	mg/day	0 1 0 4
Copper	mg/day	0 1 0 5
Fluorine	mg/day	0 2 0 5
Iodine	mcg/day	35 75
Iron	mg/day	below 1 0
Lead	mg/day	0 05 0 5
Magnesium	mg/day	1 0 2 0
Molybdenum	mcg/day	10 30
Nickel	mcg/day	20 30
Phosphorus	gm/day	0 8 2 0
Potassium	mEq/day	20 64
Sodium	mEq/day	150 197
Solids (total)	gm/day	20 60
Sulfates (total)	gm/day	0 6 1 0
Thiocyanate	mg/day	5 8
Water	l/day	0 5 1 5
Zinc	mg/day	0 3 0 4
4 *Nitrogen and Protein Derivatives*		
Albumin	mg/day	less than 100
Alpha amino nitrogen (total)	mg N/day	300 700
Alpha amino nitrogen (bound)	mg N/day	100 200
Alpha amino nitrogen (free)	mg N/day	1 0 40
Allantoin	mg/day	5 35
Arginine	mg/day	50 150
Creatine	mg/day	less than 100
Creatinine	gm/day	1 0 1 5
Glutamine	mg N/day	10 15
Glycine	mg/day	1 0 230
Histidine	mg/day	180 250
Isoleucine	mg/day	1 0
Leucine	mg/day	0 30
Lysine	mg/day	73 100
Methionine	mg/day	8 12
Nitrogen (total)	gm/day	10 18
Threonine	mg/day	50 65
Tryptophane	mg/day	00 400
Urea	gm/day	10-40
Uric acid	gm/day	0 2 2 0
Valine	mg/day	19 25
Xanthine	mg/day	0 40
5 *Carbohydrates and Derivatives*		
Alpha ketoglutaric acid	mg/day	1-44
Citric acid	mg/day	210-4 0
Fructose	mg/day	0
Pyruvic acid	mg/day	10 25
Total reducing sugar	mg/day	0 100

TABLE 31—CONT'D

6 *Fats and Derivatives*		
Acetone bodies (total)	mg/day	10 100
Cholesterol	mg/day	0 3 1 0
7 *Vitamins*		
Ascorbic acid	mg/day	6 18
Biotin	mcg/day	10 2_
Choline	mg/day	2 4
Niacin	mg/day	0 2 1 0
N¹Methylnicotinamide	mg/day	4 1_
N Methyl _ pyridine 5 carboxylamide	mg/day	5 10
Pantothenic acid	mg/day	1 0 3.5
Pyridoxine	mg/day	0 05 0 4
Riboflavin	mg/day	0 4 1.50
Thiamine	mg/day	0 1 0 4
Vitamin A	IU/day	0
8 *Hormones*		
Androsterone	mg/day	1 2
Androgen	mg/day	3 10
Corticosterone	mg/day	0 1 0 3
Cortin	mg/day	0 _ ° 5
11 Desoxycorticosteroid	mg/day	0 1 0 5
Estrogens (female)	mcg/day	50 150
Estrogens (male)	m g/day	° 6
Glycogenic steroids	mouse units/day	40 80
17 Ketosteroids (female)	mg/day	5 14
17 Ketosteroids (male)	[illegible] day	8 °0
9 *Enzymes*		
Diastase	unit /day	8 3
Phosphatase (acid)	K ng Armstrong un ts	80 300
Trypsin	un ts	40 50
Uropepsin	unit	1000 3000
10 *Pigments*		
Coproporphyrin (Type I)	m g/day	15 90
Coproporphyrin (Typ III)	m g/day	1 24
Indican	mg/day	40 150
Urobilinogen	mg/day	0 4
Uroporphyrin	mg/day	0
11 *Blood Gases*		
Carbon d oxide	mEq/day	0 50
1 *Measurements of Clinical Interest*		
Phenols (conjugated)	mg/day	15 40
Phenols (free)	mg/day	0 0 4
Phenols (total)	mg/day	15 40

TABLE 3. COMPOSITION OF FECES

(Listed below are normal ranges for various substances in human feces all values are for healthy individuals in a resting condition at sea level in a temperate environment The values for many substances are closely related to dietary intake In addition there are large differences from person to per on)

SUBSTANCE	UNITS	USUAL RANGE
1 *Water*		
Specific gravity	vs water	1 030 1 100
Total solids	gm/100 gm	15 3,
Water	ml/100 gm	65 85
2 *Hydrogen Ion Concentration*		
Hydrogen ion concentration	pH	7 0 7 5
3 *Minerals*		
Aluminum	mg/day	1 5 ? 9
Calcium	gm/day	0 1 1 0
Chloride	mEq/day	traces except in diarrhea
Copper	mg/day	1 5 2 11
Iron	gm/day	0 7 1 0
Lead	mg/day	0 3 0 4
Manganese	mg/day	1 9 2 4
Molybdenum	mg/day	2-4
Nickel	mg/day	5 10
Phosphorus	gm P/day	0 9 1 7
Potassium	mEq/day	19 ? ?? 6
Sodium	mEq/day	traces except in diarrhea
Tin	mg/day	0 5 1 7
Zinc	mg/day	5 10
4 *Nitrogen and Protein Derivatives*		
Arginine	gm/day	1 2 1
Histidine	gm/day	0 6 0 8
Isoleucine	gm/day	1 4 2.3
Leucine	gm/day	1 8 ? 9
Lysine	gm/day	1 9 ? 9
Methionine	gm/day	0 5 0 8
Nitrogen (total)	gm/day	0 7 2 1
Threonine	gm/day	1 4 2 ?
Valine	gm/day	1.5 2 6
5 *Carbohydrates and Derivatives*		
Total reducing sugar	mg/day	0
6 *Fats and Derivatives*		
Total fat	gm/day	1 ?
Total fat	per cent by weight (dry)	10 ?5
Total fat (unsaponifiable)	per cent by weight (dry)	0 5
7 *Vitamins*		
Beta carotene	mg/day	1 7 3 3
Biotin	mcg/day	100 ?00
Niacin	mg/day	3 5 5.5
Pantothenic acid	mg/day	1 8 3 8
Pyridoxine	mg/day	0 1 0 5
4 Pyridoxic acid	mg/day	0.5-0 6
Riboflavin	mg/day	0 4 1.20
Thiamine	mg/day	0 ?-0 8
Vitamin A	mg/day	0 17-0.33
8 *Hormones*		
No data available	—	—

TABLE 3 —CONT D

9 *En ymes*		
No data available		
10 *Pigments*		
Porphyrin total	mcg/day	300 400
Protoporphyrin	mcg/day	0 300
Urobilinogen	mg/day	40 80
11 *Gases*		
No data available		
1 *Measurements of Clinical Inte est*		
Bacte ial debris	per cent of weight (dry)	10 30

TABLE 3 COMPOSITION OF FECES

(Listed below are normal ranges for various substances in human feces all values are for healthy individuals in a resting condition at sea level in a temperate environment The values for many substances are closely related to dietary intake In addition there are large differences from person to person)

SUBSTANCE	UNITS	USUAL RANGE
1 *Water*		
Specific gravity	vs water	1 030 1 100
Total solids	gm/100 gm	1[illegible] 3[illegible]
Water	ml/100 gm	6[illegible] 8[illegible]
2 *Hydrogen Ion Concentration*		
Hydrogen ion concentration	pH	[illegible] 0 7.5
3 *Minerals*		
Aluminum	mg/day	1[illegible] 9
Calcium	gm/day	0 1 1 0
Chloride	mEq/day	traces except in diarrhea
Copper	mg/day	1 5 [illegible] 11
Iron	gm/day	0 7 1 0
Lead	mg/day	0.3 0 4
Manganese	mg/day	1 9 [illegible] 4
Molybdenum	mg/day	2-4
Nickel	mg/day	5 10
Phosphorus	gm P/day	0 9 1 7
Potassium	mEq/day	19 [illegible] 6
Sodium	mEq/day	traces except in diarrhea
Tin	mg/day	0 [illegible] 1 7
Zinc	mg/day	5 10
4 *Nitrogen and Protein Derivatives*		
Arginine	gm/day	1 [illegible] 2 1
Histidine	gm/day	0 6 0 8
Isoleucine	gm/day	1 4 [illegible] 3
Leucine	gm/day	1 8 2 9
Lysine	gm/day	1 9 3 9
Methionine	gm/day	0.5 0 9
Nitrogen (total)	gm/day	0 7 [illegible] 1
Threonine	gm/day	1 4 2 2
Valine	gm/day	1.5 2 6
5 *Carbohydrates and Derivatives*		
Total reducing sugar	mg/day	0
6 *Fats and Derivatives*		
Total fat	gm/day	1 7
Total fat	per cent by weight (dry)	10 [illegible]5
Total fat (unsaponifiable)	per cent by weight (dry)	0 5
7 *Vitamins*		
Beta carotene	mg/day	1 3 3
Biotin	mcg/day	100 [illegible]00
Niacin	mg/day	3.5 5.5
Pantothenic acid	mg/day	1 8 3 8
Pyridoxine	mg/day	0 1 0.5
4 Pyridoxic acid	mg/day	0.5 0 6
Riboflavin	mg/day	0 4 1 20
Thiamine	mg/day	0 [illegible] 0 8
Vitamin A	mg/day	0 17-0 33
8 *Hormones*		
No data available		

TABLE 3.—CONT'D

9 *Enzymes*		
No data available		
10 *Pigments*		
Porphyrin total	mcg/day	300-400
Protoporphyrin	mcg/day	0 300
Urobilinogen	mg/day	40 80
11 *Gases*		
No data available		
12 *Measurements of Clinical Interest*		
Bacterial debris	per cent of weight (dry)	10 30

TABLE 32 COMPOSITIO OF FECES

(Listed below are normal ranges for various substances in human feces all values are for healthy individuals in a resting condition at sea level in a temperate environment The values for many substances are closely related to dietary intake In addition there are large differences from person to person.)

SUBSTANCE	UNITS	USUAL RANGE
1 *Water*		
Specific gravity	vs water	1 030 1 100
Total solids	gm/100 gm	15 35
Water	ml/100 gm	65 85
Hydrogen Ion Concentration		
Hydrogen ion concentration	pH	7 0 5
3 *Minerals*		
Aluminum	mg/day	1 5 °9
Calcium	gm/day	0 1 1 0
Chloride	mEq/day	traces except in diarrhea
Copper	mg/day	1 5 2 11
Iron	gm/day	0 7 1 0
Lead	mg/day	0 3 0 4
Manganese	mg/day	1 9 °4
Molybdenum	mg/day	°4
Nickel	mg/day	5 10
Phosphorus	gm P/day	0 9 1 7
Potassium	mEq/day	19 2 °° 6
Sodium	mEq/day	traces except in diarrhea
Tin	mg/day	0 5 1 7
Zinc	mg/day	5 10
4 *Nitrogen and Protein Derivatives*		
Arginine	gm/day	1 ° 1
Histidine	gm/day	0 6 0 8
Isoleucine	gm/day	1 4 ° 3
Leucine	gm/day	1 8 ° 9
Lysine	gm/day	1 9 2 9
Methionine	gm/day	0 5 0 8
Nitrogen (total)	gm/day	0 7 ° 1
Threonine	gm/day	1 4 ° 2
Valine	gm/day	1.5 2 6
5 *Carbohydrates and Derivatives*		
Total reducing sugar	mg/day	0
6 *Fats and Derivatives*		
Total fat	gm/day	1 °
Total fat	per cent by weight (dry)	10 °5
Total fat (unsaponifiable)	per cent by weight (dry)	0 5
7 *Vitamins*		
Beta carotene	mg/day	1 7 3 3
Biotin	mcg/day	100 °00
Niacin	mg/day	3 5 5.5
Pantothenic acid	mg/day	1 8 3 8
Pyridoxine	mg/day	0 1 0.5
4 Pyridoxic acid	mg/day	0 5 0 6
Riboflavin	mg/day	0 4 1 °0
Thiamine	mg/day	0 2 0 8
Vitamin A	mg/day	0 1 0.33
8 *Hormones*		
No data available		

TABLE 3°—CONT D

9 *En ymes*		
No data available		
10 *Pigments*		
Porphyrin, total	mcg/day	300 400
Protoporphyrin	mcg/day	0 300
Urobilinogen	mg/day	40 80
11 *Gases*		
No data available		
1 *Measurements of Clinical Interest*		
Bacterial debris	per cent of weight (dry)	10 30

INDEX

C

D

M

N